DESENSITISATION OF THE PITUITARY VASOPRESSIN RECEPTOR: DEVELOPMENT AND USE OF A STABLY-TRANSFECTED MODEL CELL SYSTEM TO ASSESS THE ROLE OF G PROTEIN-COUPLED RECEPTOR KINASES

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Siobhan Cummings

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TABLE OF CONTENTS

ABSTRACT

Stress impacts upon all organisms and a robust stress response is required for adaptive interactions of the organism with the environment. In most higher organisms, an individual's response to stress is mediated by the hypothalamic pituitary adrenal (HPA) axis. Inappropriate regulation of this axis can cause debilitating mental health disorders including depression and anxiety. These disorders can affect an individual's ability to interact and respond appropriately as different situations arise.

An important component of this axis is the vasopressin V1b receptor (V1bR), which mediates adrenocorticotropin (ACTH) secretion from the anterior pituitary in response to stimulation by arginine vasopressin (AVP). AVP also potentiates the ACTH secretion mediated by corticotropin-releasing hormone type 1 receptor (CRH-R1) in response to corticotropin-releasing hormone (CRH) stimulation. Both the V1bR and CRH-R1 are G protein coupled receptors (GPCRs). A common feature of GPCR signalling is desensitisation of the response following prolonged or repeated exposure to an agonist. Phosphorylation of the receptor is one of the mechanisms of desensitisation. This directly, or indirectly, results in rapid and reversible uncoupling of the receptor from its heterotrimeric guanine nucleotide binding protein (G-protein). Previous research has shown that G protein coupled receptor kinases (GRKs) are key phosphorylators involved in the molecular mechanism of GPCR desensitisation. One of the mains goals of the research carried out in the Mason laboratory is to examine the molecular mechanisms of V1bR desensitisation. The current short term aim is to examine the potential role for GRKs in this mechanism.

It is difficult to study a single receptor type and the molecular mechanisms involved in its regulation in a system larger than a cell based assay. As the proposed method of assessing the involvement of GRKs in desensitisation of the V1bR is to use RNA interference (RNAi) to knock down the expression of the GRKs, primary cell cultures of pituitary corticotrophs are an inappropriate choice. This is due to a number of factors, including the difficulty involved in transfecting primary cells, and the difficulty involved in interpreting the results from primary cell culture experiments as these cultures are composed heterogenous population of cells. Therefore, the main aim of this research was to develop a model cell system from an immortalised cell line, stably-transfected with the V1bR, in which the involvement of GRKs in the molecular mechanism of V1bR desensitisation could be studied. Development of

stably-transfected cell lines requires substantial preliminary work and planning in order to produce a successful outcome. Once developed, characterisation of the clonal cell lines is required.

The preliminary work involved determining the cell proliferation rate of the parental cell line, plasmid sub-cloning and production of a large quantity of plasmid DNA, optimisation of the antibiotic selection conditions, and optimisation of the transfection protocol, as well as modification of the inositol phosphate (IP) assay protocol. The V1bR activates the phospholipase Cβ (PLCβ) second messenger signalling pathway in response to stimulation with AVP. This results in the production of IPs and therefore, measurement of IPs in response to AVP stimulation of cells labelled with *myo*-[^{3}H]inositol can be used as an indicator of functional V1bR expression.

In this research a total of nine clonal cell lines resistant to the antibiotic G418 were generated. Initial testing of these lines indicated that four probably expressed the V1bR and these were selected for characterisation in greater detail. All of these four lines showed significantly increased IP production in response to AVP stimulation ($P<0.05$; *t*-test). A significant decrease in IP production in response to AVP stimulation following an AVP pre-treatment was also seen with all four lines ($P<0.05$; *t*-test). Current evidence therefore suggests that the V1bR in these clonal cell lines signals and desensitises in the normal way. Although further characterisation of the clonal cell line is desirable, the data to date indicate that these lines should be considered to provide an appropriate model system for examining the molecular mechanisms involved in the regulation of the V1bR. It appears that there are some minor differences in signalling between the clonal cell lines and therefore this should be a consideration when deciding which line is most appropriate to use for investigating a particular question.

LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin
ATCC	American Type Culture Collection
AVP	arginine vasopressin
B_2AR	β_2-adrenergic receptor
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CK1α	casein kinase 1α
CPM	counts per minute
CRH	corticotropin releasing hormone
CRH-R1	corticotropin releasing hormone receptor type 1
DAG	diacylglycerol
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
E. coli	*Escherichia coli*
ER	endoplasmic reticulum
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EQ1	equilibration buffer
EU	endotoxin unit
F6	FuGENE 6
FBS	foetal bovine serum
g	gravitational force or grams (depends on context)
G-protein	heterotrimeric guanine nucleotide binding protein
G418	geneticin
GOI	gene of interest
GPCR(s)	G-protein coupled receptor
GRK(s)	G-protein coupled receptor kinase
h	hour(s)
HCl	hydrochloric acid
HEK293	human embryonic kidney 293

HPA	hypothalamic-pituitary-adrenal
IP	inositol phosphate
IP_1	inositol 1-phosphate
IP_2	inositol 1,4-bisphosphate
IP_3	inositol 1,4,5-trisphosphate
kb	kilobase(s)
L	liter
L7	lysis buffer
LiCl	lithium chloride
LVP	lysine-vasopressin
M	molar
MEM	minimal essential medium
min	minute(s)
ml	millilitre(s)
mRNA	messenger ribose nucleic acid
N3	precipitation buffer
ng	nanogram
NIH	National Institutes of Health
nM	nanomolar
RNA	ribose nucleic acid
OTR	oxytocin receptor
OXT	oxytocin
PBS	phosphate buffered saline
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLCβ	phospholipase Cβ
POMC	pro-opiomelanortin
PP2B	protein phosphatase 2B
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
R3	resuspension buffer
RNA	ribose nucleic acid
RNAi	RNA interference

rpm	revolutions per minute
rRNA	ribosomal RNA
rV1bR	rat vasopressin 1 b receptor
s	second(s)
siRNA	small interfering RNA
SQPI	spectral quench parameter for ^{3}H
SV40	simian virus 40
TBE	Tris-borate/Na_2EDTA Electrophoresis buffer
T/E	trypsin/EDTA
TE8	Tris-Na_2/EDTA buffer at pH 8.0
TM	transmembrane
tRNA	transfer RNA
u	unit
µl	microlitre(s)
µg	microgram(s)
µM	micromolar
µm	micrometer(s)
V1aR	vasopressin 1a receptor
V1bR	vasopressin 1b receptor
V2R	vasopressin 2 receptor
W8	wash buffer

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1:

INTRODUCTION

1.1 REGULATION OF ACTH SECRETION

1.1.1 THE HPA AXIS

The HPA axis is an endocrine system which is utilised by higher organisms to ensure the maintenance of homeostasis in response to shboth physical and emotional stress events (Aguilera, 1994; Buckingham et al., 1997; O'Conner, 2000). Adaptive responses to stress events are essential to the survival of the organism and a maladaptive response can decrease survival chances and can cause an increased susceptibility to a variety of diseases and disorders. Specifically, disregulation of the HPA axis has been shown to be a risk factor in many mental health disorders (Pompili et al., 2010), particularly in mood disorders (Bosch et al., 2007; Carpenter et al., 2007; Parker et al., 2003; Tyrka et al., 2008; Veenema, 2009).

The general outline of the HPA axis is depicted in Figure 1.1. As can be seen activation of the HPA axis ultimately results in the production of glucocorticoids, which regulate a wide range of targets to ensure an adequate stress response and the maintenance of homeostasis (Aguilera, 1994; Buckingham et al., 1997; O'Conner, 2000). Glucocorticoid release is regulated by the hormone ACTH, which is released from corticotroph cells in the anterior pituitary gland. Regulation of ACTH secretion is a tightly controlled multi-factorial process (Aguilera, 1994; Antoni, 1993). The hypothalamic neuropeptides, CRH and AVP, are the two most potent secretagogues involved in ACTH release. CRH and AVP are secreted into the hypophyseal portal circulation from the hypothalamus in a pulsatile fashion. Stimulation of ACTH secretion by CRH and AVP occurs when the peptides bind to their specific GPCRs. CRH binds to the CRH-R1, and AVP to the V1bR. It is believed that in some species, including rats and primates, CRH controls and maintains the basal level of ACTH secretion as well as stimulating the production of the ACTH pre-cursor molecule pro-opiomelanortin (POMC) (Aguilera, 1994; Gillies et al., 1982; Krieger, 1983), with AVP potentiating ACTH secretion, particularly in response to stress events. Release of both CRH and AVP from the hypothalamus is increased in response to stress events. This release is differentially regulated in response to different types of stress, which allows the stress response to be tailored

appropriately to the situation (Aguilera, 1994). As stated above CRH and AVP stimulate the release of ACTH, while negative feedback from secreted glucocorticoids inhibits both the release of ACTH from the pituitary and CRH and AVP from the hypothalamus (Buckingham et al., 1997). ACTH also regulates the secretion of CRH and AVP from the hypothalamus through negative feedback.

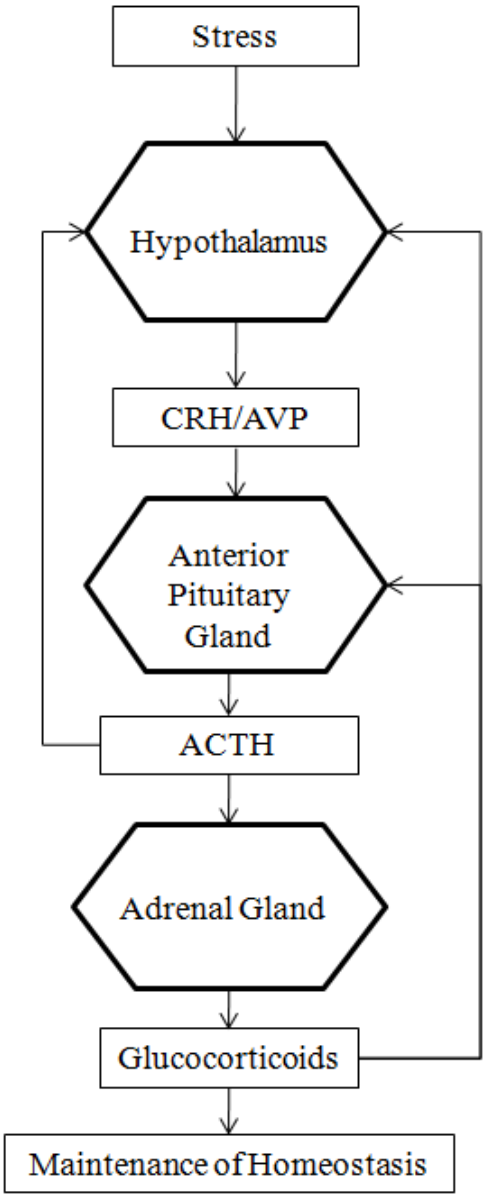

Figure 1.1 The HPA axis. The series of steps in the HPA axis pathway are depicted. Both ACTH and glucocorticocoids are sources of negative feedback, which helps to regulate the axis. The figure is adapted from Buckingham et al., (1997).

Commonly GPCRs, like CRH-R1 and V1bR, are also regulated through negative feedback within the cell, which causes the response to become desensitised as a result of repeated or prolonged agonist stimulation (Ferguson, 2001; Lohse, 1993). It is believed that the regulation

of the receptor content of the V1bR maybe an important step in the control of the ACTH responsiveness of pituitary corticotrophs and therefore, the adaptation to stress (Aguilera et al., 1994). A key mechanism of GPCR desensitisation is phosphorylation of the receptor, which uncouples it from its signalling pathway (Lohse, 1993). This is a rapid and reversible mechanism of desensitisation. Other mechanisms of desensitisation include the internalisation and down regulation of the receptor, but these occur over longer time scales (Ferguson, 2001). The V1bR has been shown to be desensitised at physiologically relevant concentrations and durations of AVP stimulation of ovine pituitary cells (Hassan et al., 2003). However, despite some investigation into the molecular mechanisms of V1bR desensitisation (Hassan and Mason, 2005) it still remains to be fully elucidated.

1.1.2 THE ROLE OF AVP IN THE REGULATION OF ACTH SECRETION

AVP is a neuropeptide hormone containing nine amino acids and is very closely related to oxytocin (OXT) (Barberis et al., 1998; Vincent and Su, 2008). AVP is synthesised by two groups of neurons in the hypothalamus, the parvocellular cells in the paraventricular nucleus and magnocellular neurons in the supraoptic and paraventricular nuclei (Antoni, 1993). Axons of the parvocellular system project into the external zone of the median eminence, where they secrete AVP into pituitary portal secretion. The magnocellular system secretes AVP into the peripheral circulation from axon terminals in the neural lobe of the pituitary. Evidence suggests that the parvocellular neurons are the primary source of AVP responsible for ACTH regulation in response to stress (Aguilera, 1994).

AVP has been implicated in the release of ACTH from the anterior pituitary since the 1950's (for an example see Martini and Morpurgo, 1955), however, it is only relatively recently that the importance of its role in the HPA axis has become clear. Most previous work involving ACTH secretion has focused on the role of CRH, as it was known to be a potent secretagogue for ACTH secretion and thought to most probably be the main secretagogue in most animals (Antoni, 1993). It is now known that AVP potentiates CRH stimulated ACTH secretion and that the stimulation of the pituitary by both peptides results in a synergistic response (Gillies et al., 1982). It has been proposed that for a number of species, including primates and rats, that the CRH system maintains basal secretion of ACTH and stimulates the production of POMC mRNA while AVP stimulation is crucial in the response to stress, particularly chronic stress (Aguilera, 1994; Aguilera and Rabadan-Diehl, 2000b; Antoni, 1993). Although, research in mice models has indicated that the AVP/V1bR system is involved with both the basal and stress responses of the HPA axis (Tanoue et al., 2004). Work in ovine anterior

pituitaries has shown that the ACTH response is desensitised following an AVP pre-treatment at physiologically relevant concentrations and durations (Hassan et al., 2003). This suggests regulation of the ACTH response to AVP stimulation is a physiologically relevant process.

1.1.3 MECHANISMS OF AVP STIMULATED ACTH RELEASE IN THE ANTERIOR PITUITARY

1.1.3.1 AVP stimulated receptors

As mentioned in Section 1.1.1, the effects of AVP are mediated through GPCRs. Like all GPCRs, the AVP receptors share the common structural features of seven transmembrane (TM) α-helices, with an extracellular amino terminus and an intracellular carboxyl terminus (Kobilka, 2007). The most variable parts of the GPCR structure are in the amino- and carboxyl- termini and the third intracellular loop, between TM5 and TM6. The amino terminus of GPCRs is variable in length, to allow for accommodation of a wide variety of agonists. For GCPRs activated by peptides, like the AVP receptors, the amino terminus is usually between 10-50 amino acids long. The carboxyl terminus and the third intracellular loop are known to be involved in coupling with G-proteins. GPCRs are generally very hard to crystallise due to their transmembrane portions and their inherent flexibility. However, crystal structures have been obtained for rhodopsin and these structures have been used to elucidate most of the structural information available for GPCRs (Kobilka, 2007; Palczewski et al., 2000). Other methods have been used to elucidate structural detail from other GPCRs as well, including site directed mutagenesis, cysteine scanning mutagenesis, and engineered metal ion binding sites (Kobilka, 2007). However, as rhodopsin is easy to use and readily available it has made this the GCPR of choice for investigations into structure.

The flexibility that makes GPCRs so hard to crystallise is generally considered to be functionally important, in that it allows the receptors to specifically alter their conformations in response to agonist binding (Ferguson, 2001; Kobilka, 2007). These conformational changes mainly occur in the TM segments and cytoplasmic region of the receptor. Most commonly the relative positions of TM3, TM5, and TM6 are altered, which results in re-orientation of the third intracellular loop. The agonist stabilises these changes and therefore the partially or fully active conformational states. The agonist binding to the receptor induces the conformational changes through activation of specific combinations of molecular switches. Once activated the GPCR couples to a specific G-protein. The G-protein transduces the signal from the receptor to an intracellular second messenger system.

As well as mediating ACTH secretion through the V1bR, AVP also has a number of other physiologically relevant effects which it mediates through the other GPCRs that it specifically activates. There are a total of four different receptors which AVP can activate, including the V1bR. There are two other specific VP receptors, the vasopressin 1a receptor (V1aR), and the vasopressin 2 receptor (V2R), as well as the single oxytocin receptor (OTR) (Vincent and Su, 2008). These receptors show a high level of sequence homology, however, they do bind AVP in different ways, but with similar binding affinities (Rodrigo et al., 2007; Thibonnier et al., 2002; Vincent and Su, 2008). The V1aR, the V1bR, and the OTR, signal via coupling to $G_{q/11}$ and therefore PLCβ, while the V2R signals via coupling to G_s and therefore adenylyl cyclase (reviewed in Maybauer et al., 2008). In terms of physiological roles, the V2 receptor is present in the kidney and mediates the antidiuretic activity of AVP (reviewed in Maybauer et al., 2008). The V1aR is present across the widest range of bodily tissues, with its most significant roles in the mediation of vasorestriction and social behaviour (reviewed in Maybauer et al., 2008; Shepard et al., 2009). As mentioned above the V1bR mediates the release of ACTH from the anterior pituitary, although it is also present in a wide range of other tissues, where it mediates a variety of other physiological roles (Lolait et al., 1995; Maybauer et al., 2008; Saito et al., 1995; Vaccari et al., 1998).

Using bovine, human, mouse, and rat AVP receptors and combinations of cell culture and membrane preparation techniques along with mouse and rat animal models, selective agonists (Derick et al., 2002; Pena et al., 2007) and antagonists (Serradeil-Le Gal et al., 2002) have been and are being used to more fully elucidate the differences between the receptors (Guillon et al., 2004). Due to the similarities between the receptors, many of the synthetic agonists which have been designed to be V2 specific agonists are also partial V1bR agonists, which causes problems when they are used medically (Guillon et al., 2006). Therefore, in order to design more effective pharmaceuticals the differences between the receptors need to be fully characterised so that pharmaceutical specificity can be increased. There are also some species specific differences seen with receptor binding affinities and pharmacological properties with regards to the synthetic agonists and antagonist (Chini and Manning, 2007; Guillon et al., 2004; Guillon et al., 2006; Pena et al., 2007; Serradeil-Le Gal et al., 2002; Thibonnier et al., 2002), which could have implications for extrapolation of signalling details from one species to another.

1.1.3.2 The V1bR

The V1bR is a GPCR with 424 amino acids in humans (De Keyzer, 1994; Sugimoto et al., 1994), and 421 amino acids in rats (Lolait et al., 1995). It was shown to be a distinct receptor structurally related to the AVP/OXT superfamily that functionally couples to PLCβ (De Keyzer, 1994; Sugimoto et al., 1994). It binds to AVP in a manner distinct from that of the other AVP receptors (Thibonnier et al., 2002). The amino acid sequence of the receptor contains consensus sequences for both protein kinase C (PKC) and GRKs.

V1bR mRNA has been identified in a number of rat tissues including the thymus, heart, lung, spleen, kidney, uterus, breast, small intestine, liver, pancreas, and in a number of areas in the brain (Lolait et al., 1995; Saito et al., 1995; Vaccari et al., 1998). In the brain the V1bR is present in greater amounts in the forebrain, hypothalamus, amygdala, and cerebellum and less in mid and hindbrain regions (Hernando et al., 2001; Vaccari et al., 1998). The distribution of the V1bR in the brain is distinct from both the distributions of the OTR and V1aR, which are also present in the brain, indicating functional specialisation of the receptors. This wide distribution contributes to the wide variety of physiological responses that have been determined for the V1bR, including the release of insulin (Pena et al., 2007), catecholamines (Guillon et al., 1998) and glucagon (Yibchok-Anun et al., 1999). The receptor has been shown to have a role in regulation of cell proliferation (Thibonnier et al., 1997), water balance, and body temperature (Daikoku et al., 2007). There are also indications of involvement of the receptor in social interactions and emotional responses such as aggression (Caldwell and Young III, 2009; Egashira et al., 2009; Wersinger et al., 2002), social motivation (Wersinger et al., 2004) and social recognition (Wersinger et al., 2002). Most of this research determining the physiological roles of the V1bR has utilised null mice models. However, some rat models have been used as well, along with human and rat perifused tissue.

1.1.3.2.1 Signalling of the V1bR

There are a large number of extracellular residues that appear to be involved in the receptor binding of AVP (Barberis et al., 1998). Therefore, it has been speculated that the hormone/receptor complex is an intricate network of hydrogen bond interactions. The role of arginine-8 of AVP is particularly important in binding to the V1bR, where it interacts with the negatively charged amino acids in the receptor binding sites (Rodrigo et al., 2007). A proposed model of AVP binding to the V1bR can be seen in Figure 1.2.

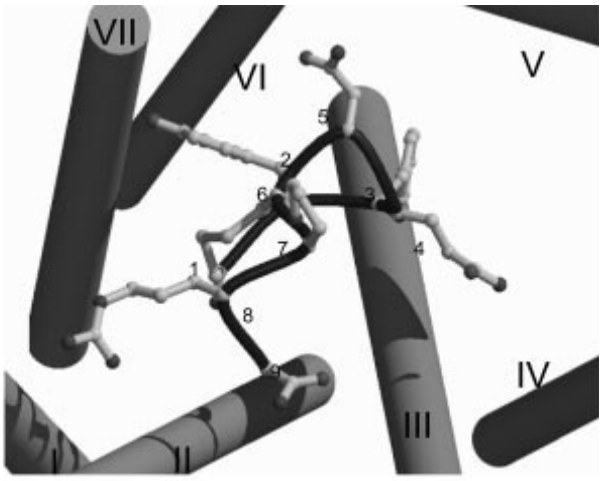

Figure 1.2 AVP binding to the V1bR. A three dimensional image of the proposed binding (Rodrigo et al., 2007).

Once AVP has bound to the V1bR, normal signal transduction is achieved by coupling of the receptor to the G-protein, $G_{q/11}$ (Alberts et al., 2002; King and Baertschi, 1990; Maybauer et al., 2008; Thibonnier et al., 1997). $G_{q/11}$ works by activating PLCβ which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates release of Ca^{2+} from intracellular stores, particularly from the endoplasmic reticulum (ER), through binding to gated Ca^{2+} channels. DAG, along with the increase in intracellular Ca^{2+}, activates protein kinase C (PKC) which phosphorylates target proteins within the cell as well as stimulating entry of Ca^{2+} from the extracellular space. This increase in free intracellular Ca^{2+} levels stimulates the release of ACTH from the cell. Figure 1.3 depicts this general signalling pathway. The Ca^{2+} release activated by IP_3 is responsible for the spike phase of the response which is seen initially. The Ca^{2+} release activated by DAG is responsible for the sustained response (reviewed in Mason et al., 2002).

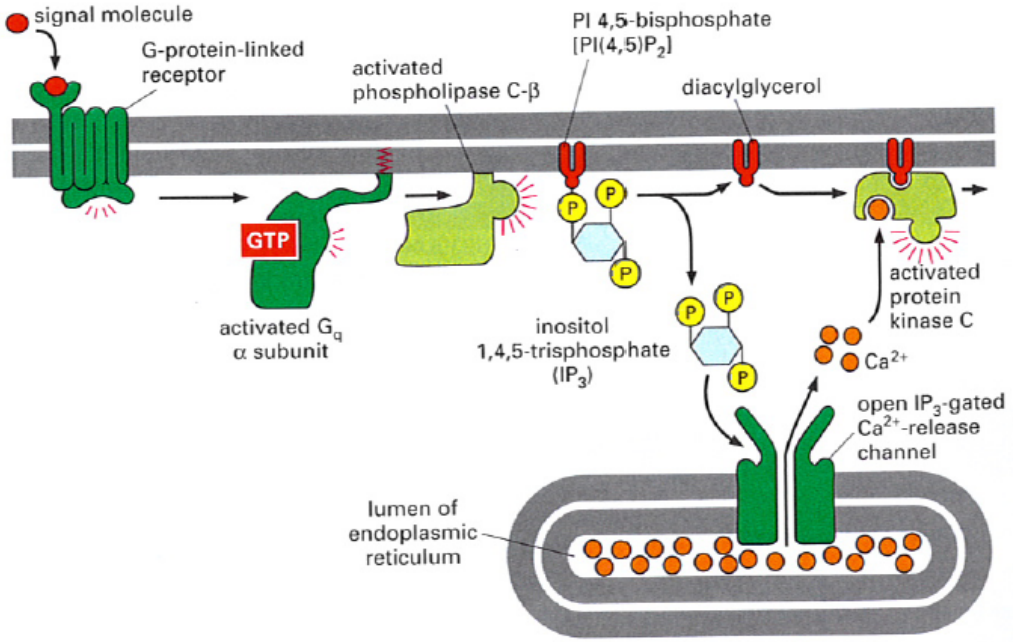

Figure 1.3 Intracellular signalling of GPCRs coupled to $G_{q/11}$. The agonist binds to the GPCR activating it. Once activated it binds to the G-protein $G_{q/11}$. This causes the α-subunit of the G-protein to separate from the βγ-subunit. Once separated from the βγ-subunit, the α-subunit activates PLCβ. The activated PLCβ then hydrolyses PIP_2 to IP_3 and DAG. IP_3 stimulates the release of Ca^{2+} from intracellular Ca^{2+} stores. DAG along with this increase in intracellular Ca^{2+} activate PKC (Alberts et al., 2002).

V1bR signalling is affected by the number of receptors expressed in the membrane and by the concentration of AVP available (Orcel et al., 2009; Thibonnier et al., 1997). Low levels of receptor expression in Chinese hamster ovary (CHO) cells resulted primarily in the activation of PLCβ signalling (as described above), and high levels of receptor expression in these cells resulted in activation of both PLCβ and adenylyl cyclase signalling pathways (Thibonnier et al., 1997). In HEK293 and COS-7 cells transiently transfected with the V1bR higher concentrations of AVP were able to produce signalling through both PLCβ and adenylyl cyclase, whereas lower concentrations could only elicit signalling through PLCβ (Orcel et al., 2009). This occurred in cells with both a normal and a higher than normal level of receptor expression. When the cells were stimulated with the selective V1bR agonist d[Cha^4]vasopressin signalling occurred through both signalling pathways at equivalent potencies.

The V1bR can form homo-dimers as well as hetero-dimers with the CRH-R1 and OTR (Young et al., 2007). Current indications are that formation occurs in the ER and is a non-regulated process. Dimerisation has been seen among many different GPCR families but is only necessary for receptor activation in a few (Kobilka, 2007). Evidence suggests that signalling with these V1bR dimers can happen differentially through $G_{q/11}$ and G_s (Orcel et

al., 2009), potentially making this an important process in the normal signalling of the cells. The presence of these dimers has created the potential to design bivalent analogues for assessing the receptors and these have therapeutic possibilities (Chini and Manning, 2007). Signalling depends upon the ligand as well as the localisation of the receptors within the plasma membrane. Localisation within different plasma membrane domains has been shown to affect signalling via PLCβ coupled GPCRs (Golebiewska and Scarlata, 2010).

1.1.3.2.2 The role of the V1bR in ACTH secretion

As mentioned above the major physiological role for the V1bR is in the anterior pituitary, where it mediates ACTH secretion in response to AVP simulation (reviewed in Maybauer et al., 2008). Several studies, including both *in vitro* experiments, with rats, mice, ovine, and equine anterior pituitary cells, and *in vivo* studies with rats, have shown that ACTH responsiveness to AVP is decreased following prolonged exposure (reviewed in Mason et al., 2002). The regulation of ACTH responsiveness to AVP is thought to be important in the HPA axis adaptation to stress, particular for maintaining ACTH responsiveness during chronic stress (Aguilera, 1994; Aguilera and Rabadan-Diehl, 2000b; Antoni, 1993). Evidence from a number of studies involving rats suggests that AVP plays a key role in regulating V1bR expression and as a consequence the ability of the pituitary to respond to activation of the HPA axis (Aguilera and Rabadan-Diehl, 2000a). Several different chronic stress paradigms were examined with rats to investigate the relationship between V1bR receptor levels and AVP stimulated ACTH release (Aguilera et al., 1994). The results from these experiments showed a direct relationship between ACTH responsiveness and V1bR levels during chronic stress. This suggests a role for receptor regulation in adaptations of the HPA axis to chronic stress. Work in ovine pituitaries has suggested that the receptor regulation is a physiologically relevant process (Hassan et al., 2003).

Both transcriptional (Rabadan-Diehl et al., 2000; Volpi et al., 2002) and translational (Rabadan-Diehl et al., 2007; Rabadan-Diehl et al., 2003) mechanisms have been indicated to be involved in the regulation of the rat V1bR expression. However, it appears that it is the translational mechanisms that are key for regulation of the total receptor content of cells (Aguilera et al., 2003; Volpi et al., 2004). There are two key regulatory elements in the 5' untranslated region which appear to be involved in translational V1bR regulation. These are repressor small open reading frames (Nomura et al., 2001; Rabadan-Diehl et al., 2007) and an up-regulating internal ribosomal entry site (Rabadan-Diehl et al., 2003).

It has recently been suggested that regulation of the V1bR may not directly mediate the hypersensitivity of ACTH responses in chronic stress paradigms, and instead maybe regulating cell proliferation and remodelling of the pituitary tissue (Aguilera et al., 2008). There is evidence in support of this theory, indicating that AVP has a mitogenic effect and that this can be mediated by the V1 type receptors (Tahara et al., 1999; Van Wijk et al., 1995). Whether or not receptor regulation directly affects ACTH secretion, information on the molecular mechanisms controlling the regulation of the V1bR should still prove valuable.

1.2 DESENSITISATION OF THE ACTH RESPONSE TO AVP

When activated by an agonist GPCRs transduce a signal through conformational changes which couple the receptor to a signalling pathway (Kobilka, 2007) (see Section 1.1.3.1 for details). These conformational changes also enable the process of desensitisation, which is a physiologically relevant regulatory mechanism which limits acute and chronic over stimulation (Ferguson and Caron, 1998). GPCRs desensitise in response to either repeated or prolonged exposure to their agonist.

In ovine pituitary cells desensitisation of the ACTH response to AVP stimulation (100 nM, 5 min) has been shown to occur following pre-treatment of the cells for 5 min with 5 nM AVP (Hassan et al., 2003). Pre-treated cells showed a significant decrease in ACTH response to 78.5% of the response seen in control cells that were not pre-treated. An AVP pre-treatment of this concentration and duration falls within endogenous ranges in the sheep. This indicates that the process of V1bR desensitisation is likely to be a physiologically relevant process and therefore it is likely to be a key process in the regulation of ACTH release *in vivo*.

1.2.1 MECHANISMS OF DESENSITISATION OF GPCRs

Desensitisation of GPCRs can occur by a number of mechanisms including, uncoupling of the receptor from its signalling pathway through receptor phyosphorylation, internalisation of the receptor, and down regulation of the total receptor content (Ferguson, 2001). These mechanisms occur over different time scales. Uncoupling of the receptor from its signalling pathway results in a rapid and reversible form of desensitisation (Ferguson, 2001; Lohse, 1993). Phosphorylation is generally a complex and specific process, which allows for differential signalling outcomes (Kelly et al., 2008). Phosphorylation of specific GPCRs has been shown to be catalysed by one, or a number of, protein kinases, including protein kinase

A (PKA), protein kinase B (PKB), protein kinase C (PKC), the G-protein coupled receptor kinase (GRK) family, the casein kinase family, and tyrosine kinases (Lohse, 1993; Tobin, 2008). Which kinase(s) is specifically involved depends upon the receptor and in some cases the agonist that is activating the receptor, which indicates that different functional conformations of the receptors are phosphorylated by different kinases (Bailey et al., 2009).

Phosphorylation of receptors by GRKs promotes the binding of *β*-arrestins, which not only leads to uncoupling of the receptor from its cognitive G-protein, but also targets the receptor for internalisation (Ferguson, 2001; Kelly et al., 2008). Once internalised the receptors are then either, dephosphorylated, re-sensitised, and returned to the plasma membrane, or they are degraded and the total cellular complement of the receptor is down regulated. This classical model of desensitisation is depicted in Figure 1.4.

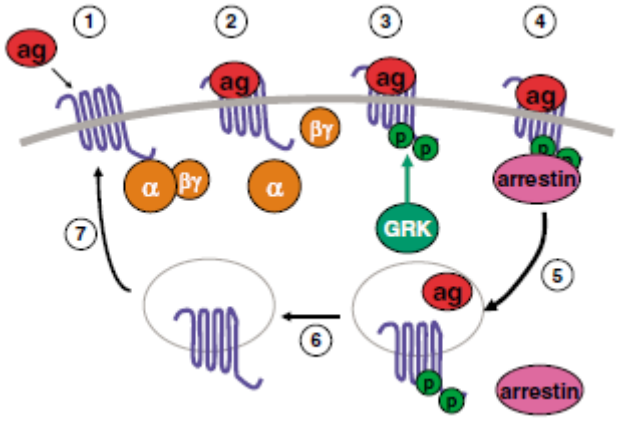

Figure 1.4 The classical model of GPCR signalling, as regulated by GRKs and *β*-arrestins. The GCPR is activated by the agonist (1) which results in the coupling of the receptor to a specific G-protein, activating it (2) this leads to the separation of the α-subunit from the βγ-subunit. The activated receptor is phosphorylated by GRK (3), arrestin binds to this phosphorylated receptor, which leads to receptor desensitisation (4), internalisation (5), dephosphorylation (6) and recycling back to the plasma membrane (7). GPCRs which have been internalised may also be targeted for down regulation (Kelly et al., 2008).

Most of the information currently known about the desensitisation of GPCRs has been elucidated using β_2-adrenergic receptor (β_2AR) (Ferguson, 2001). However, the classical desensitisation mechanism outlined is thought to be similar for all GPCRs (Ferguson, 2001; Mason et al., 2002; Tobin, 1997), including receptors coupled to the PLCβ signalling pathway which have been shown to undergo rapid desensitisation. The magnitude of the

desensitisation mediated by phosphorylation is variable between PLCβ coupled receptors (Wojcikiewicz et al., 1993).

A number of factors can affect GPCR signalling and desensitisation including compartmentalisation of GPCRs within different membrane domains (Golebiewska and Scarlata, 2010; Russo et al., 2009), dimerisation both homo- and hetero-, (Kobilka, 2007), and different receptor conformations (Wei et al., 2003). Some GPCRs are still able to signal in endosomes after internalisation, however, this signalling can have different functional consequences from signalling which occurs at the plasma membrane (Calebiro et al., 2010). There is also evidence indicating that there is a phosphorylation-independent pathway for the attenuation of GPCR signalling as well as the phosphorylation-dependent pathway (Ferguson, 2007).

1.2.1.1 The classical role of GRKs in GPCR desensitisation

GRKs are a family of serine/threonine protein kinases that specifically bind to and phosphorylate agonist occupied GPCRs (Ferguson, 2001; Menard et al., 1997). There are seven different GRKs which can be divided into three different groups: 1) GRKs -1 and -7, 2) GRKs -2 and -3, and 3) GRKs -4, -5, and -6 (Ferguson, 2001; Pitcher et al., 1998; Premont et al., 1995; Reiter and Lefkowitz, 2006). GRKs -1, and -7 are found exclusively in the retina, and GRK4 expression is mainly confined to the cerebellum, testes and kidneys. All of the other GRKs are ubiquitous in mammalian tissues. GRK1 was the first GRK to be identified and is specifically associated with rhodopsin. GRKs -2 and -3 were identified through their involvement with the β_2AR and are therefore also referred to as β_2-adrenergic-kinase -1 and -2 respectively. GRKs1-3 are located in the cytosol under normal conditions, these GRKs are then translocated to the plasma membrane upon activation of the receptor. GRKs 4-7 are located at the plasma membrane. GRK4 and GRK6 are associated with the membrane via palmitoylation on carboxyl-terminal cysteine residues. GRK5 membrane association is thought to be mediated by electrostatic interactions of highly basic amino acids in the carboxyl-tail of the protein with membrane phospholipids. GRKs phosphorylate GPCRs at serine and threonine residues located within the third intracellular loop or the carboxyl-terminal domain of the receptors.

There appears to be a level of redundancy among GRKs; when one GRK is knocked down or out another can generally compensate for its absence. Knockout of GRK2 is the only GRK knock out which is embryonically lethal in mice (Pitcher et al., 1998), indicating that there is

some functional specificity. Specifically there are indications that the different groups of GRKs have different roles within the signalling process and in some cases compete with each other (Reiter and Lefkowitz, 2006). For example GRKs -2 and -3 have been shown with the angiotensin II receptor to be more efficient in activating β-arrestin, clathrin dependent endocytosis (Kim et al., 2005). Different cell types will also express different levels of the various GRKs, providing an ability for different tissues to tailor regulation of the same receptor to specific signalling purposes, thereby enabling a variety of physiological roles for the same receptor (Tobin, 2008). This may be the case with the V1bR which has been shown to have a wide variety of physiological roles.

1.2.2 MECHANISM OF V1bR DESENSITISATION: CURRENT KNOWLEDGE

As the V1bR is a GPCR, it is thought that it might follow a similar model of desensitisation to that described in Section 1.2.1. However, the specifics of the molecular mechanisms involved require investigation. Previous work in this laboratory, carried out using ovine anterior pituitary cells has provided data indicating that neither PKC or casein kinase 1 α (CK1α) are involved in the desensitisation process (Hassan and Mason, 2005). The data also indicated that protein phosphatase 2B (PP2B) is involved in the re-sensitisation process. PP2B has been shown with the β_2AR to reverse desensitisation mediated by GRK2 (Shih et al., 1999). Therefore, it seems that the most likely mediator(s) of V1bR phosphorylation is a GRK(s). The V1bR amino acid sequence contains consensus motifs for GRKs (Mason et al., 2002; Thibonnier et al., 2002), which confirms the potential ability of these kinases to mediate V1bR phosphorylation. The possible involvement of GRKs in the desensitisation process of the V1bR is therefore a current focus of research in this laboratory. The role of GRKs in the mechanism of V1bR desensitisation is currently being assessed using RNAi, as there are no pharmalogical inhibitors of specific GRKs. The difficulties associated with using RNAi techniques in primary cell cultures have necessitated the development of a model cell system.

1.3 USE OF A MODEL CELL SYSTEM TO INVESTIGATE THE MECHANISM OF V1bR DESENSITISATION

A wide variety of experimental methods, including both cellular studies and genetically altered animal models, have been employed previously to assess the role of specific GRKs in the phosphorylation of a wide variety of GPCRs (Pitcher et al., 1998). RNAi is the method of choice for loss of function studies, due to its relative efficiency and efficacy compared with

other techniques (Editorial, Nature Cell Biology, 2003). This approach has therefore been chosen in the Mason laboratory to assess the role of individual GRKs in desensitisation of the V1bR. However, the use of RNAi has necessitated a move away from primary cell cultures of ovine anterior pituitaries (Hassan, 2001; Hassan et al., 2003) to the development of a model cell system using an immortalised cell line (Gatehouse, 2008). The reasons for this are varied and include the following. Firstly, primary cell cultures of mature ovine anterior pituitaries divide infrequently, if at all, in a cell culture environment, whereas immortalised cell lines will divide continuously under the right conditions, making them more practical for long term studies (Freshney, 2000; Grimm, 2004; van Beijnum et al., 2008). Secondly, immortalised cell cultures are also composed of a phenotypically and genetically uniform population that have been derived from a specific tissue, whereas primary anterior pituitary cell cultures are composed of a heterogenous population of cells. For example the anterior pituitaries contain corticotroph cells which express the V1bR. The OTR is also expressed in the anterior pituitary, but within other cell types. The presence of another receptor activated by AVP which signals via PLCβ in the anterior pituitary will make any results from biochemical assays measuring levels of intracellular molecules after AVP stimulation very difficult to interpret. Thirdly, transfection efficiency is limited in primary cells, which means that they can be difficult to use with RNAi techniques.

1.3.1 HEK293 CELLS TRANSIENTLY TRANSFECTED WITH THE V1bR AS A MODEL CELL SYSTEM

Previous work in the Mason laboratory (Gatehouse, 2008) examining desensitisation of the V1bR has used human embryonic kidney (HEK)293 cells transiently transfected with the rat V1bR (rV1bR) gene (Lolait et al., 1995), as a model cell system. This cell line was first described in 1977 (Graham et al., 1977), and is extensively used in research. In fact, as of January 2011, the original paper has been cited over 3,000 times according to the ISI Web of Knowledge. HEK293 cells were chosen for this research for a number of reasons, including the fact that this cell line is an excellent recipient of transfected DNA (Graham, 1992) and is widely used in research for this purpose. In an in-depth microarray analysis of HEK293 cells (Shaw et al., 2002) (see accompanying database http://www.mbi.ufl.edu/~shaw/293.html) there was no indication of expression for any of the AVP receptors or the OXT receptor. Experiments in this laboratory (Gatehouse, 2008) and others (Orcel et al., 2009) have shown that there is no increase in IP production in response to a saturating concentration of AVP (100 nM) without transfection of an AVP receptor. This confirms that no AVP activated

receptors are normally expressed in HEK293 cells. Therefore, HEK293 cells are a suitable recipient for the rV1bR. A human cell line, like the HEK293 line, is a good choice for RNAi work as the sequences of human GRKs are known and siRNA duplexes to target them have been published and used to examine the role of GRKs in signalling via the angiotensin II receptor (Kim et al., 2005; Wei et al., 2003), the V2 receptor (Ren et al., 2005), and the β_2AR (Violin et al., 2006). Therefore, these factors make HEK293 cells a good choice as the model system for this research.

When using transient transfections the level of expression of the transfected gene can vary with each transfection event. Generation of a V1bR stably-transfected cell line can minimise this problem. Over long term experiments the production of stably-transfected cell lines is significantly less costly and time consuming than repeated transient transfections.

1.3.2 THE GENERATION OF A V1bR STABLY-TRANSFECTED MODEL CELL SYSTEM: REQUIREMENTS TO ACHIEVE THIS

The main aim of this research was to develop a model cell system stably expressing the rV1bR, in order to avoid the problems associated with transient transfections that were described above. Transfection of cells is said to be stable when there is continued expression of the gene over time (Grimm, 2004), for example when the plasmid DNA containing the gene is integrated into the cell's genome.

Generation of clonal stably-transfected cell lines improves consistency between experiments including consistent protein expression levels and signalling characteristics. Cell lines stably-transfected to express a gene of interest (GOI), provide a homogenous population of cells in which the gene and the protein produced can be analysed (Grimm, 2004; Mortensen and Kingston, 2009). Analysis of genes and proteins in a cell system closely related to the source cell system allows for a greater level of extrapolation. Mammalian cells stably expressing a variety of genes are also currently used in production of mammalian recombinant proteins for pharmaceuticals because these cells can properly assemble, fold, and modify mammalian proteins (Wurm, 2004). In order to generate stably-transfected cell lines, several factors need consideration and consequently several preliminary steps are required before the cells are transfected and selected for. These include plasmid design (Mairhofer and Grabherr, 2008), transfection reagent selection and optimisation for use with a specific cell line (Mortensen and Kingston, 2009), and the selection and optimisation of a selection

marker. Once these stably-transfected cell lines have been generated a suitable method of analysis of the expression of the GOI is required.

An important step in developing a cell system which will express a specific protein stably, is to determine which method of plasmid delivery should be used as DNA is a large, highly charged molecule and therefore cannot cross the plasma membrane without help (Bonetta, 2005). There are a wide variety of reagents that can be used for the introduction of DNA into mammalian cells, each with its own advantages and disadvantages that need to be evaluated (Bonetta, 2005; Grimm, 2004; Mortensen and Kingston, 2009). The differences between the methods affect the mechanism used to get the DNA into the cytosol and nucleus as well as affecting the way in which the plasmid DNA is integrated and expressed. Therefore, this can affect the expression profiles obtained from the stable cell line. Methods of introducing DNA to mammalian cells include carrier molecules, electroporation, and viruses. Introduction of DNA via carrier molecules is termed transfection, whereas viral introduction is termed transduction (Bonetta, 2005).

Carrier molecule methods of DNA introduction are generally straightforward and efficient to use (Bonetta, 2005). There is also a wide variety of different reagents to choose from (Bonetta, 2005). The various types of carrier molecules will encapsulate/complex with the DNA. This complex is then believed to be incorporated into the cell through endocytosis (Ausubel et al., 1994; Felgner et al., 1987; Grimm, 2004). The DNA is then released from the endosome, but how this is achieved by the complex is currently not well understood. Plasmid design can be used to facilitate importation into the nucleus, however, importation is generally most efficiently achieved during cell division when the nuclear membrane ruptures (Vaughan et al., 2006). Therefore, these methods tend to work best on dividing cells. Carrier molecule methods usually introduce the plasmid DNA to the chromosome as multiple copies at a single site (Mortensen and Kingston, 2009). This usually creates a tandem array of similar sequences within the host's genome. The presence of these arrays can lead to intra-chromosomal rearrangement over time, resulting in a drop in copy number, which will lead to a decrease in the levels of expression.

Electroporation uses an electrical current to create transient holes in the plasma membrane of cells (Ausubel et al., 1994; Bonetta, 2005). Once a hole has been created there is no longer a barrier to DNA entry, and therefore it can access the cytosol with ease. However, despite the wide applicability of this method, it is very harsh on cells and use generally results in high

levels of cytotoxicity. In order to use this method expensive equipment is required. This method will also work best on dividing cells (Vaughan et al., 2006). Electroporation will most commonly introduce one copy of the DNA at one site in the host's genome (Mortensen and Kingston, 2009).

Introduction of DNA using viruses is the most efficient method of introduction, as the viruses have naturally evolved proteins which are highly efficient at facilitating entry to the cell and in some cases the nucleus (Bonetta, 2005; Grimm, 2004). However, the protocols can be time consuming and their use presents bio-safety issues. Retroviruses contain the protein, integrase, which they can use to insert the plasmid DNA into the host cell's genome, making them a very useful tool for developing stable cell lines. The only draw-back to this is that only the lentivirus genus of this family of viruses, for example the human immunodeficiency virus, can infect non-dividing cells, the rest of the retrovirus family must infect dividing cells. The other virus family commonly used for transduction are adenoviruses and although these do not have their own proteins for facilitating DNA integration into the genome they can introduce DNA to a wide range of cells with high efficiencies.

Integration of the DNA into the genome is believed to be a random event whether introduction to the cell was mediated by chemical or viral methods (reviewed in Murnane et al., 1990). DNA has been shown to integrate via both homologous and non-homologous recombination pathways (Crabb and Cowman, 1996; Murnane et al., 1990). Viral methods use virus protein to incorporate the plasmid DNA into the host's genome. Whereas with chemical methods it is believed that the cellular machinery is used to facilitate integration via DNA repair and recombination enzymes (Haber, 1999).

The random nature of DNA integration can produce difficulties in the generation of stable cell lines, as many of these integration events do not result in adequate expression of the GOI (Mortensen and Kingston, 2009). To address this issue, it is possible to utilise homologous recombination to introduce DNA at specific sites. This is mainly done when creating transgenic animals, but it can also be used for stable cell line creation. One such system that can be utilised is a *lox* recombination vector, which is designed to directly select Cre-mediated site-specific DNA integration at a *lox* target site previously incorporated into the genome (Fukushige and Sauer, 1992). Cre is a recombinase protein that mediates site specific integration between *loxP* sites, on the plasmid and in the genome. Systems like this produce a

reproducible level of expression. This is not a suitable approach to obtain stable cell lines if the functional impact of differing levels of expression is of interest.

Carrier molecule and electroporation methods are suitable for both introduction of DNA on a single plasmid or on two separate plasmids (co-transfection) (Ausubel et al., 1994; Mortensen and Kingston, 2009). Carrier molecule protocols are less complicated than ones for viruses and less equipment is required than for electroporation (Bonetta, 2005). This makes carrier molecular methods suitable for inexperienced users and a suitable choice for a laboratory where only a small number of transfections is required.

Selection and maintenance of expression using a dominant selection marker provides a simple method of isolating stable cells from those with only transient expression (Mortensen and Kingston, 2009). There are number of options when choosing a dominant selection marker, and one must be chosen that will produce the desired outcome within the timeframe available.

An appropriate assay must be set up to assess the functioning of the transfected gene (Grimm, 2004). This is a key step, as before the cell line is used further it must be assessed to ensure that gene expression is normal. Characterisation of the expression levels of the gene are also crucial and required before further work with the cell line is undertaken.

1.3.2.1 Plasmid design to improve stable integration of plasmid DNA and protein expression

Several factors in plasmid design can help to increase transfection efficiency (Mairhofer and Grabherr, 2008). These include a reduction in the size of the plasmid to improve delivery, protection from degradation while in the cytosol, targeting to the nucleus, and minimisation of prokaryotic elements within the plasmid.

Successful transfection is highly dependent upon the cell cycle, with gene expression 50-300 times higher when cells are transfected just before G2 phase or during mitosis (reviewed in Hebert, 2003; Vaughan et al., 2006). This is because the nuclear envelope ruptures and the plasmid gains unimpeded access to the nucleus. This method of gaining access to the nucleus is utilised most commonly when transfecting dividing cells. However, when transfecting non-dividing cells the plasmid must be targeted and imported into the nucleus, this process is enhanced by a nuclear localisation signal (reviewed in Nakanishi et al., 2001). In general nuclear import is believed to be an active signal-mediated process which occurs via the nuclear pores. There are a number of transport pathways that can be utilised and these are

mediated by a variety of carrier proteins within the cell. For example plasmids containing specific sequences from simian virus (SV)40 have been shown to enhance nuclear targeting and import (Dean, 1997; Dean et al., 1999; Nakanishi et al., 2001; Vaughan et al., 2006). The majority of the plasmid DNA imported with this SV40 sequence, co-localized with the SC-35 splicing complex antigen which suggests that, in this case at least, that the nuclear import of the DNA was dependent on transcriptional processes.

Once the DNA has reached the nucleus, expression is affected by the site of DNA integration into the chromatin (Wurm, 2004). Integration into inactive condensed heterochromatin can result in little to no expression of the gene, whereas integration into active euchromatin allows for a greater level of expression. Typically in mammalian cells about 10% of the genome is packaged into heterochromatin (Alberts et al., 2002). Flanking transgenes with protective *cis*-regulatory elements such as insulators, boundary elements, scaffold/matrix attachment regions, ubiquitous chromatin opening elements and conserved antirepressor elements can help reduce the negative effects of heterochromatin on expression (reviewed in Wurm, 2004).

1.3.2.2 Selection of an appropriate transfection reagent

Selection of an appropriate transfection reagent is necessary to ensure that the desired results are achieved for expression of the stably-transfected gene (Hunt et al., 2010; Mortensen and Kingston, 2009). Carrier molecules have been the method of choice in the Mason laboratory for the introduction of DNA into HEK293 cells previously (Gatehouse, 2008). This is because they provided a relatively straight forward and low cost method of DNA introduction with relatively low cytotoxicity in mammalian cells (Bonetta, 2005). FuGENE 6 (F6) is a carrier molecule transfection reagent and was chosen as the transfection reagent for this project due to its ease of use and low cytotoxicity, especially with HEK293 cells (Bonetta, 2005; Jacobsen et al., 2004). F6 is a non-liposomal formulation of proprietary compounds that has been shown to successfully transfect a wide range of cell lines (Bonetta, 2005). In order to obtain the best results, several parameters of the transfection protocol need to be optimised for use with the chosen cell line (see Section 3.4)

1.3.2.3 Selecting for stably-transfected HEK293 cells

When generating a stably-transfected cell line a dominant selection marker is required, as it provides the cells which have stably-integrated the plasmid with a selective advantage (Mortensen and Kingston, 2009). In this research Geneticin®, also known as G418, was used to select for the stably expressing cells. G418 provides a means to select cells if a G418

resistance gene has been transfected along with the GOI (Southern and Berg, 1982). Aminoglycoside 3'-phosphotransferase (APH(3')II) from transposon Tn5, designated as *neo*, is the gene which provides mammalian cells with resistance to G418 (Davies and Jimenez, 1980). G418 was originally isolated from *Micromonospora rhodorangea* (Wagman et al., 1974), and is similar in structure to other aminoglycoside antibiotics, such as gentamicin, neomycin, and kanamycin, which all act on prokaryote cells (Southern and Berg, 1982). Aminoglycoside antibiotics affect prokaryote cells by binding to the aminoacyl-tRNA site (A site) on 16S ribosomal RNA, thus decreasing the accuracy of translation in the cells (Vicens and Westhof, 2003). G418 works as a selective agent in eukaryote cells by preventing protein synthesis through interference with the 80S ribosome (Davies and Jimenez, 1980), specifically the 18S subunit (Kaul et al., 2005). G418 will cause the de-stacking of base 1492, and this de-stacking in the rRNA site is the critical factor in the action of the antibiotic. In order to successfully use a selection agent in the selection of stable clones the minimal concentration needed for killing the parental cell line must be determined (see Section 3.3). This ensures that the concentration determined will be lethal to all cells that do not integrate the *neo* gene, allowing for those which do stably-integrate the gene into their genomes to be selected for (Southern and Berg, 1982).

1.3.2.4 Characterisation of the developed stable cell lines

Characterisation of stable cell lines is important to determine the level of expression of the GOI and whether the protein produced is functioning normally (Grimm, 2004). Once the cell line have been characterised, lines with high and low levels of gene expression can be identified. Full characterisation of cell lines is useful when analysing results obtained with a stable cell line and also when comparing with other cells expressing the gene.

Characterisation of GPCRs, can be achieved through measurements of molecules produced within the signalling pathway in response to agonist stimulation. The V1bR is coupled to $G_{q/11}$ which activates the PLCβ to hydrolise PIP_2 to IP_3 and DAG. In turn these molecules stimulate an increase in intracellular Ca^{2+} (see Section 1.1.3.2). Measurement of IP accumulation (Gatehouse, 2008; Rabadan-Diehl et al., 2007; Thibonnier et al., 1997; Young et al., 2007) and changes in intracellular Ca^{2+} levels (Gu and Lee, 2010; Nezu et al., 2010; Sato et al., 2009; Young et al., 2007) are two potential ways of assessing functional signalling with $G_{q/11}$ coupled GPCRs. Receptor binding assays, with [^{3}H]AVP can be utilised to determine the actual numbers of V1bRs present at the cell surface and the binding properties (Aguilera et al., 1994; Young et al., 2007).

Measurement of IP accumulation is a widely used method for assessing signalling in $G_{q/11}$ coupled GPCRs. After the hydrolysis of PIP_2 to IP_3 and DAG, IP_3 is de-phosphorylated in a stepwise manner (Berridge et al., 1983), and then joins up with DAG metabolites and eventually regenerates PIP_2. Li^+ inhibits the enzyme inositol monophosphatase preventing the de-phosphorylation of inositol 1-phosphate (IP_1) to inositol (Berridge et al., 1982). This causes the IP_3, 1,4-bisphosphate (IP_2), and IP_1 to accumulate within the cell. The total IPs can then be separated from other water soluble cell extracts using anion exchange chromatography and increasing concentrations of formate as described previously (Downes and Michell, 1981; Richards et al., 1979). By radio-labelling the cells with *myo*-[^{3}H]inositol prior to agonist stimulation and subsequent IP extraction and separation, the eluted samples can be counted using a liquid scintillation counter (Berridge et al., 1983).

This method can be used to assess the function signalling of any GPCRs which signal via PLCβ, and has been used previously to assess the V1bR signalling (Gatehouse, 2008; Rabadan-Diehl et al., 2007; Thibonnier et al., 1997; Young et al., 2007). Critically the IP assay can be used for the assessment of GRK involvement in GPCR desensitisation (Pitcher et al., 1998). For example it has been used in the investigation of the role of GRK2 in desensitisation of the metabotrophic glutamate 1 A receptor using transiently transfected HEK293 cells (Dale et al., 2000).

1.4 AIMS OF THIS RESEARCH

The aims of this research were the following:

1) generate and screen HEK293 cell lines stably-transfected with the rV1bR,
2) characterise at least one of these cell lines,
3) develop various methodologies required to meet the above objectives.

The main goal of this research was to develop an rV1bR stably-transfected clonal, model-cell system that could be used for investigating regulation of the receptor at the molecular level. The successful development of a cell line stably expressing a GOI requires the optimisation of various methodologies. Once a model cell system is developed, characterisation of its functional profile and the expression level of the protein of interest are required before the system can be used for further research. For this model cell system future research will

involved investigations into the molecular mechanism involved in the desensitisation of the V1bR.

Due to the developmental nature of this thesis, the results section has been divided up into two chapters. Chapter 3 deals with the experimental work and results that were required for preparation and generation of the stable cell lines. Chapter 4 deals with the initial characterisation of V1bR expression within these cell lines.

CHAPTER 2:

STANDARD METHODS

2.1 MATERIALS

For the sources of materials used in this research please refer to Appendix A.

2.2 SOLUTIONS AND MEDIA

For the details of all solutions and media used in this research please refer to Appendix B.

2.3 CELL CULTURE

2.3.1 STANDARD CELL CULTURE CONDITIONS

In this research HEK293 cells were either cultured untransfected or they were transiently or stably transfected with plasmids containing either the enhanced green fluorescent protein (EGFP) or the rV1bR. The plasmids used for transfection also contained the *neo* gene unless specified otherwise. Details of both of these plasmids can been found in Section 2.5.1.1. Figure 2.1 shows (untransfected) HEK293 cells growing normally.

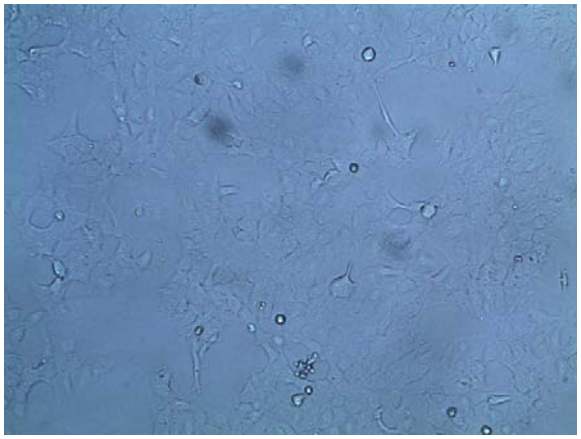

Figure 2.1 Normal growth of HEK293 cells. Under normal growth conditions HEK293 cells have a neuronal morphology, and the monolayer of cells is flat against the tissue cultureware. All images of HEK293 cells, unless otherwise stated, were taken on a Leica DM IL microscope using a x20 objective lens and a x10 eyepiece. This allowed for direct comparison between images.

The HEK293 cells were cultured in Eagle's Minimal Essential Medium (MEM) (Invitrogen), containing Earle's salts, L-glutamine, and non-essential amino acids, with sodium bicarbonate (BDH Laboratory Supplies) and sodium pyruvate (Invitrogen) added (full MEM details in Appendix B). After three weeks, the remaining MEM was supplemented with GlutaMAX™ (Invitrogen), due to the breakdown of L-glutamine over that time period. For standard culturing, the medium was supplemented with 10% (v/v) foetal bovine serum (FBS) (Invitrogen). This supplemented medium will be referred to from here on as MEM+, and any departure from this formulation will be indicated.

For standard culturing HEK293 cells were grown in either 75 cm^2 or 25 cm^2 flasks in 15 ml or 5 ml of MEM+ respectively. The size of the flasks used varied depending on the number of cells required for specific experiments. Plates with a standard cell cultureware coating were used for all experiments unless specified otherwise. The cells were cultured at 37°C inside a CO_2 water-jacketed incubator (IR Autoflow, Nuaire) in a humid environment of 95% air, 5% CO_2.

All procedures and experiments involving the HEK293 cells were carried out in a Biological Safety Cabinet-Class II (BH2000, Clyde-Apac), to ensure the maintenance of aseptic conditions. Only sterile reagents came into contact with the cells. All plastic ware was received sterile from suppliers. All solutions were obtained already sterilised, or those made in the laboratory were sterilised by filtration (through 0.22 μm filters or in the case of culture medium through 0.45 μm filters) or autoclaving (121°C, 20 min, 20 lb/in^2). Glassware was sterilised either by autoclaving or dry heat sterilisation (2 h, ≥170°C).

2.3.2 SUB-CULTURING OF HEK293 CELLS

The HEK293 cells were regularly sub-cultured (passaged) at 50-80% confluence. It is important for the maintenance of the functional viability of HEK293 cells, to ensure that they do not reach 100% confluence or greater, as this negatively impacts growth, morphology, and function of the cells in later passages. Cells were not passaged when they were below 50% confluence as this caused cell growth to be slowed in subsequent passages.

To passage cells in a 75 cm^2 flask, the MEM+ was removed via aspiration and 3 ml of 0.25% trypsin with 1 mM ethylenediaminetetraacetic acid (EDTA) (T/E) (Invitrogen) pre-warmed to 37°C was added to the flask. The flask was tilted to allow the T/E solution to cover the entire cell monolayer, and the T/E was then swiftly removed. The cells were incubated at room temperature for 8 min, during which time they were observed on an inverted microscope

(TMS, Nikon). During incubation with T/E, HEK293 cells lose their adherence to the flask and dissociate from one another. The morphology of the cells changes from the typical neuronal morphology seen with these cells to a round morphology. At the end of 8 min, the flask was tapped twice on the bottom and twice on the side. Four to eight ml (for standard passaging) or ten ml (for freezing cells, see Section 2.3.4) of MEM+ pre-warmed to 37°C was then added to the flask with a 5 ml or 10 ml plastic pipette so that the medium washed down over the cells, dislodging them from the surface of the flask. The cell suspension was re-pipetted six times, to ensure the break up of any cell clumps, and removed to a 50 ml centrifuge (Falcon) tube. The cells were then counted (see Section 2.3.3) and a volume of cell suspension containing 0.7 x 10^6 cells (to passage next in 3 days) or 0.4 x 10^6 cells (to passage next in 4 days) was added to a 75 cm^2 flask containing 15 ml of MEM+ pre-warmed to 37°C. The cells were cultured for 24 h to allow for adherence. At this point the medium was replaced, to ensure the removal of any traces of T/E. The medium was replaced again during the cell culture period if it changed colour, indicating a change in pH. Cells were maintained for no more than ten passages. Only low passage-number cells were used for cryopreservation (see Section 2.3.4). If passaging a 25 cm^2 flask, all values were reduced to one third of the total values given above.

2.3.3 CELL COUNTING

The cells were counted using a haemocytometer to ensure that accurate cell numbers were used for both experiments and passaging. This accuracy increased predictability in the experimental results and cell growth. The cell counts were also used to determine the viability of the cells with trypan blue exclusion (see Appendix B for solution details). Cell viability was typically between 95-100%.

2.3.4 FREEZING AND STORAGE OF CELLS BY CRYOPRESERVATION

Cryotubes containing HEK293 cells, prepared as described below, were stored in liquid nitrogen. Cells were frozen immediately after trypsinisation (see Section 2.3.2). The 50 ml tube, containing the cell suspension, was centrifuged at 150 x g for 8 min at room temperature (CR 4-12, Jouan). The supernatant was aspirated, and then the bottom of the tube was tapped forcefully to loosen the cell pellet. For cells from a 75 cm^2 flask split at ~80% confluence, four 1 ml aliquots were made for freezing in either Recovery™ Cell Culture Freezing Medium (Invitrogen) or freezing medium that was made in the laboratory just prior to freezing (for details see Appendix B). To do this 4 ml of chilled Recovery™ Cell Culture Freezing Medium

was added to the pellet and used to re-suspend the cells, or 2 ml of MEM without additives was added and used to re-suspend the cells followed by 2 ml of freshly made freezing medium. If a different sized flask was split, requiring a different number of tubes to be frozen, the volumes were adjusted. For example, when freezing the cells from a 25 cm^2 flask at ~80% confluence, the volumes of media were halved. The freezing mediums were added and the cells re-suspended using sterile 5 ml plastic pipettes. The cells were re-pipetted as few times as possible to minimise cell damage while still ensuring an even suspension. Aliquots (1 ml) of the cell suspension were transferred to labelled Cryo.S tubes (Greiner Bio-one), and placed into a Nalgene Cryo 1° Freezing Chamber containing isopropyl alcohol, to ensure gradual freezing of the cells. The freezing unit was then transferred to a -80°C freezer. Twenty-four hours later the cryo-tubes were moved from the -80°C freezer to liquid nitrogen storage. The cells were kept in liquid nitrogen storage until required for use.

2.3.5 THAWING OF CRYOPRESERVED CELLS

To thaw the HEK293 cells a tube of cells were removed from liquid nitrogen storage and thawed immediately in a 37°C water bath with gentle shaking. The thawed cell suspension was transferred using a 5 ml plastic pipette to either a 75 cm^2 flask or a 25 cm^2 flask containing either 19 ml or 4 ml of pre-warmed MEM+ respectively. The flask was then placed into the CO_2 incubator for culturing. After 24 h of culturing, to allow the cells to adhere to the flask, the medium was replaced with 15 ml or 5 ml, respectively, of fresh MEM+ to remove the freezing medium and any non-adherent cells from the flask. The medium was also replaced on subsequent days if the MEM+ had changed colour.

2.4 MEASUREMENT OF HEK293 CELL PROLIFERATION RATE

2.4.1 DETERMINATION OF PROLIFERATION RATE

To determine the proliferation rate of HEK293 cells, the cells were plated at two densities (1 x 10^4 cells/well and 3 x 10^4 cells/well), into the wells of two 24 well plates (Thermo Fisher Scientific), in 1 ml of MEM+. The cells were counted at 24 h intervals over a period of 10-12 days by lifting the cells in two replicate wells at both densities. An estimate of the average confluence of the cells at each plating density was also made at 24 h intervals.

To lift the cells for counting, the medium was removed from duplicate wells by aspiration and 75-200 µl T/E was added to each well depending on the confluence (see Table 2.1). The

plates were incubated for 12 min at 37°C in a CO_2 incubator. By incubating the plates in the incubator rather than at room temperature, it minimised disturbance to the cells in the wells which were not being lifted at that time point. At the end of the incubation a volume of MEM+, equal to the volume of T/E used, was added to each well and the cell suspension was re-pipetted 7-11 times using a P1000 Gilson pipette set to 80% of the total volume of T/E plus MEM+. The re-pipetted cell suspensions were immediately removed to labelled 4 ml polystyrene tubes and the 24 well plates returned to the CO_2 incubator. The cells were then counted as indicated in Section 2.3.3 and the total number of viable cells/well calculated.

The medium was replaced in the wells when the colour changed from standard, indicating a change in pH. The medium was removed and added using a P1000 Gilson pipette. All cell counts were carried out at the same time each day, to ensure standard 24 h time intervals over the growth period.

Confluence	Vol. T/E
0% - 40%	75µl
40% - 60%	100µl
60% - 80%	125µl
80% - 95%	150µl
95% +	200µl

Table 2.1 Volume of T/E required to dissociate HEK293 cells at different confluences. Greater volumes of T/E were required to disassociate HEK293 cells from the culture ware with increasing confluence. All volumes given are for 1 well of a 24 well plate.

2.4.2 ANALYSIS OF PROLIFERATION DATA

The daily count data from each plating density were plotted on a semi log graph. An individual sigmodial dose-response (variable slope) curve was fitted to each of the data sets. The doubling time was determined for both of the initial plating densities during their log phase of growth. To do this, the time it took for the cell number to increase from 0.1×10^5 to 0.2×10^5 was determined. The data were analysed and the graph produced using GraphPad Prism 4 (see Section 3.1.2) for the results.

2.5 PREPARATION OF PLASMID DNA FOR TRANSFECTION

2.5.1 SUB-CLONING OF THE rV1bR GENE INTO A SUITABLE VECTOR (pEGFP-N1) FOR STABLE TRANSFECTION

2.5.1.1 Restriction enzyme digestion of pEGFP-N1 and prV1bR

The plasmid prV1bR contains the GOI, rV1bR, as well as a gene which provides resistance to the antibiotic chloramphenicol. prV1bR is a pALTER®-MAX plasmid (Figure 2.2) sub-cloned with rV1bR. pEGFP-N1 (Figure 2.3) contains the *egfp* gene as well as the *neo* gene, which provides mammalian cells with resistance to G418. The GOI was transferred into pEGFP-N1 with the removal of EGFP. This was done to incorporate the GOI on the same plasmid as a selection marker for mammalian cells.

The plasmids prV1bR and pEGFP-N1 were digested with restriction enzymes at positions before and after the rV1bR and EGFP genes in order to remove them from their respective plasmids. It was preferable, to ensure simple ligation, that the same restriction enzymes were used to digest both plasmids. The restriction enzymes chosen were SalI and NotI. This further simplified the digestion as both enzymes work in the same buffering system. A restriction enzyme digest with a total volume of 20 µl was made up for each plasmid (Table 2.2). Both digests were prepared by adding the solutions to separate microfuge tubes from largest volume to smallest. The tubes were tapped to mix the solutions, and then centrifuged for 1-2 s to ensure the total volume was at the bottom of each tube. The tubes were then incubated in a water bath at 37°C for 1.5 to 2 h to allow for plasmid digestion.

prV1bR	pEGFP-N1
13 µl sterile H_20	13 µl sterile H_20
3 µl prV1bR (total ~1 µg)	3 µl pEGFP-N1 (total ~1 µg)
2 µl 10x Buffer O	2 µl 10x Buffer O
1 µl SalI (10 u/µl)	1 µl SalI (10 u/µl)
1 µl NotI (10 u/µl)	1 µl NotI (10 u/µl)

Table 2.2 Composition of restriction enzyme digests. The table shows the composition of the restriction enzyme digests for both prV1bR and pEGFP-N1. Both digests were made up to a total of 20 µl. The restriction enzymes and Buffer O are sourced from Fermentas.

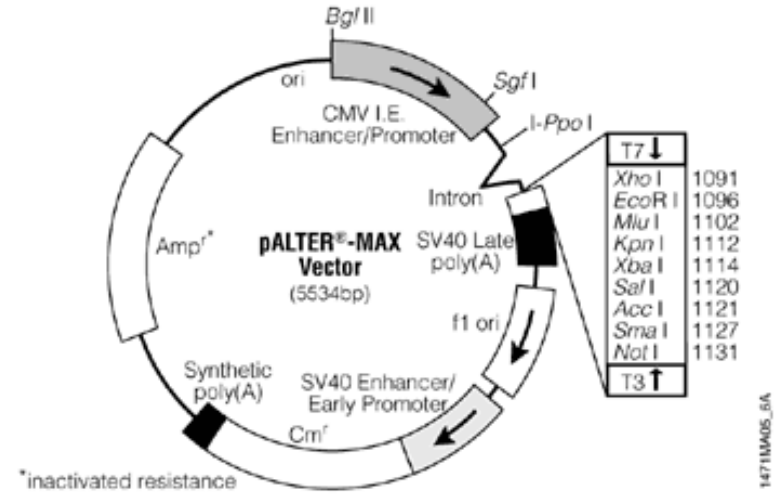

Figure 2.2 Plasmid map of pALTER®-MAX. GenBank Accession # AF316302**.**

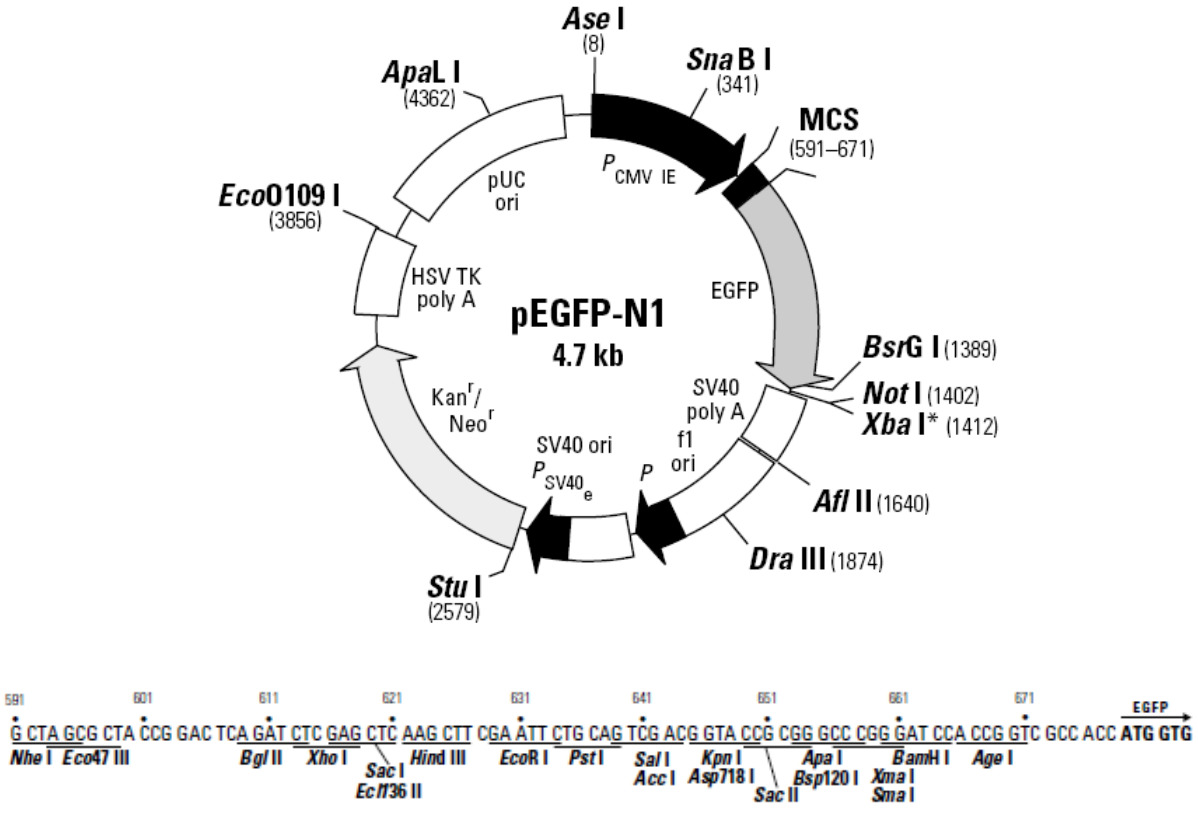

Figure 2.3 Plasmid map of pEGFP-N1. GenBank Accession # U55762. The sequence of the multiple cloning site is indicated below the plasmid map.

2.5.1.2 Separation of digestion products by gel electrophoresis

The restriction enzyme digests of prV1bR and pEGFP-N1 were run on a gel so that the digested portions of the plasmids could be separated. Once the components were separated, the ones required for sub-cloning, rV1bR and the pEGFP-N1 vector without the *egfp* gene (referred to from here on as pN1), could be removed from the gel and ligated to form the new plasmid.

An agarose (0.7%) gel in 0.5x tris borate EDTA (TBE) buffer (see Appendix B) was made. The agarose was melted and 20 µl of SYBR® Safe DNA gel stain 10,000x (Invitrogen) (1:10,000 dilution) was added. The gel was then poured into the casting tray so that it covered about 5 mm of the comb, and left to set. The restriction enzyme digest tubes were removed from the water bath and 2 µl of loading dye (supplied with ladder – Fermentas) was added to each tube. Each of the digests plus a ladder, Lambda DNA/PstI Marker, 24 (Fermentas) (10 µl), were loaded into individual wells. The wells at the edge of the gel and the wells between the individual samples were left empty, to ensure the samples remained separate. This ensured that the bands could be removed cleanly when excised from the gel. The gel was run (using a EPS 1001 power pack from Amersham Pharmacia Biotech Inc.) at 106 V for ~30 min with 0.5x TBE as the running buffer. The gel was then examined using UV illumination (DarkReader™ Transilluminator, Clare Chemical Research) to ensure clear separation of the bands and allow individual bands to be excised from the gel. Bands for the rV1bR gene (~2.6 kb) and the pN1 vector (~4.0 kb) were excised from the gel using a single edged razor blade. Once removed from the gel, the band fragments were place into labelled 1.7 ml microfuge tubes to be cleaned up prior to ligation.

2.5.1.3 Clean up and ligation of rV1bR and pN1 excised from gel

The excised gel fragments were cleaned up to remove agarose, salts, stain, and other contaminants using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corporation). Agarose Dissolving Buffer (300 µl) was added to each tube and the tubes were incubated at 55°C for 5 min or until all of the gel was dissolved. The melted gel solutions were transferred into separate Zymo-Spin™ I Columns inside 2 ml collection tubes. These were centrifuged (5424, Eppendorf) at 12,000 x g for 1 min. After centrifugation, 200 µl of Wash Buffer was added to each Column, these were then centrifuged again at 12,000 x g for 1 min. This step was repeated and the collection tubes were discarded. The Columns were placed into 1.7 ml microfuge tubes and 10 µl of sterile water was added directly to the matrix of each Column.

These were left to sit for 1 min and then centrifuged at 12,000 x g for 1 min. These steps produced ultra-pure DNA, ready for ligation. On average this Kit produces DNA at a concentration of 10 ng/µl, therefore this was the assumed concentration of the DNA used for ligation. For ligation of the insert and destination vector a 20 µl ligation solution (see Table 2.3) was made containing a 3:1 ratio of insert:vector (this gives an ~4.5:1 molar ratio). This ratio ensured that the destination vector ligated with the insert. The ligation solution was left to incubate at room temperature for 1.5 h.

13 µl sterile H_2O
3 µl insert (rV1bR) (total ~30 ng)
2 µl 10x T4 DNA Ligase Buffer
1 µl destination vector (pN1) (total ~10 ng)
1 µl T4 DNA Ligase (5 u/µl)

Table 2.3 Ligation solution for rV1bR and pN1. The Ligation Buffer and T4 DNA Ligase were sourced from Fermentas.

2.5.1.4 Transformation of competent *E. coli* cells with the ligated plasmid in order to amplify it

Competent DH5 alpha *E. coli* cells were transformed with the ligated plasmid and cultured. This was so that plasmid preparations could be made from individual colonies, in order to assess the success of the sub-cloning. The cultures of *E. coli* containing successfully sub-cloned plasmids were then used to create glycerol stocks.

A 100 µl aliquot of frozen, competent DH5 alpha *E. coli* cells was thawed on ice and split into two separate 1.7 ml microfuge tubes each containing 50 µl. Ligation solution (see Table 2.3) (5 µl) was added to each tube. To get the *E. coli* to take up the plasmid DNA the tubes were left on ice for ~2 min, heat shocked at 37°C for ~2 min, placed on ice for a further 2 min and then 200 µl of LB Broth Miller (Becton Dickson) was added to each tube. The *E. coli* suspension from each tube was plated on two agar plates (see Appendix B): 150 µl onto the first plate, and 50 µl onto the second. The use of two agar plates, plated with different volumes of bacterial suspension, ensured that individual colonies could be picked. The plates contained 30 µg/ml of the selection antibiotic, kanamycin (see Appendix B for stock solution details). This ensured that only the *E. coli* transformed with *neo* would be able to grow. The

plates were incubated overnight at 37°C to allow time for the transformed *E. coli* to grow into colonies. The next morning the plates were examined and colonies noted.

2.5.1.5 Harvesting and purification of plasmid DNA

The plasmid DNA synthesised by the *E. coli* cells needed to be harvested and purified. After which, it could be used for assessment of sub-cloning. This assessment was required to ensure that the desired plasmid was available for the generation of V1bR-stably expressing HEK293 cells.

Ten McCartney bottles, each containing ~10 ml of LB Broth Miller (Becton Dickson) were set up and kanamycin stock solution was added to each bottle to give a final concentration of 30 µg/ml. Individual *E. coli* colonies were picked from all four plates using a yellow pipette tip and placed into separate bottles. These bottles were then incubated overnight with shaking (at 250 rpm) at 37°C (ROSI 1000™, Thermolyne). The following morning, ~1.5 ml of culture was poured from each bottle into separate 1.7 ml microfuge tubes and centrifuged at 12,000 x g for 2 min. The supernatant was discarded and another ~1.5 ml of culture from the same bottle was poured into the same tube and centrifuged again at 12,000 x g for 2 min. This provided a large enough cell pellet for harvesting the plasmid DNA. The AxyPrep™ Plasmid Miniprep Kit (Axygen Biosciences) was used to harvest and purify the plasmid DNA. Buffer S1 (250 µl) containing RNase A was added to each tube and the pellet was re-suspended by re-pipetting. Buffer S2 (250 µl) was then added to each tube and mixed by inversion. This buffer lysed the cells. The cell lysates were then neutralised by the addition of 350 µl of Buffer S3. The tubes were gently inverted to mix the solutions. It is important not to vortex the solutions when mixing them as this can cause shearing of the genomic DNA which can result in contamination of the plasmid DNA. The tubes were centrifuged for 5 min at 12,000 x g to clarify the lysates, and the supernatants were transferred to the assembled Columns and Receiver tubes. The Columns and Receiver tubes were centrifuged for 1 min at 12,000 x g, the contents of the Receiver tubes were discarded and the Columns and Receiver tubes were reassembled. W2 Buffer (700 µl) was added to each Column and they were then spun for 1 min at 12,000 x g. These wash steps removed contaminants from the plasmid DNA. The receiver tubes were discarded after this spin and each Column was then placed into a 1.7 ml microfuge tube. Elution Buffer (50 µl) was added to each Column. The Columns were incubated at room temperature for 2 min, and then centrifuged for 1 min at 12,000 x g,

to elute the plasmid DNA from the Column into the microfuge tube. After this last centrifugation the Column was discarded and the tubes with the plasmid DNA were capped.

2.5.1.6 Screening of plasmid DNA preparations for successfully sub-cloned constructs

In order to select the appropriate construct to use for the generation of rV1bR-stably transfected cell lines all ten of the plasmid DNA preparations were screened. To do this, aliquots of the ten plasmid DNA preparations from the separate *E. coli* colonies were digested using SalI and NotI so that the rV1bR and pN1 were separated and therefore the presence of both could be screened for.

A restriction enzyme digest master mix was made (see Table 2.4). A 5 µl aliquot of each plasmid DNA preparation was mixed with 15 µl of this master mix in a 1.7 ml microfuge tube. The tubes were tapped to mix and spun briefly to ensure that the total volume was deposited at the bottom of each tube. The tubes were incubated for 1 h at 37°C in a water bath. Loading buffer (2 µl) was added to each tube, and the digested samples were run on an agarose (0.7%) gel. The gel was examined under UV illumination to see which preparations contained both rV1bR and pN1. The preparation that appeared to have the greatest concentration of the desired sub-cloned construct was selected for use in further experiments. Glycerol stocks were made from the *E. coli* culture used to make this preparation. These stocks were made by adding equal volumes of glycerol to bacterial culture in a 1.7 ml microfuge tube. These tubes were then frozen and stored at -80°C. The successfully sub-cloned construct is referred to from here on as pN1-V1bR. An image of this gel was taken under UV illumination on the SynGene ChemiGenius2 camera using Gene Snap software (Syngene) (see Section 3.2.1.2).

121 µl sterile H_20
22 µl 10x Buffer O
11 µl SalI (10 u/µl)
11 µl NotI (10 u/µg)

Table 2.4 Restriction enzyme digest – master mix. The table indicates the volumes needed to make enough master mix for ten restriction enzyme digests, plus an extra 10%. Total volume of the master mix was 165 µl.

2.5.2 BULK PREPARATION OF PLASMID DNA

2.5.2.1 Transformation of competent *E. coli* cells with pEGFP-N1

Competent DH5 alpha *E. coli* cells (Invitrogen) were transformed with the plasmid pEFGP-N1. These transformed cells were used to grow bacterial cultures, from which a preparation of pEGFP-N1 plasmid DNA could be harvested.

The pEGFP-N1 DNA used for the transformation was diluted to 4 ng/µl in TE8 (see Appendix B). Three 1.7 ml microfuge tubes, each containing 50 µl of competent DH5 alpha *E. coli* cells, were thawed on ice. The first tube was the test tube and was transformed with pEGFP-N1, the second was a positive control and was transformed with pUC19 (Invitrogen), and the third was a negative control and remained untransformed. For the transformations, 2.5 µl of pEGFP-N1 plasmid DNA was added to the first tube, 2.5 µl of pUC19 (100 pg/µl) was added to the positive control tube, and 2.5 µl of TE8 with no DNA was added to the negative control tube. The plasmid DNA and *E. coli* cells were mixed by hand with gently rocking. To promote uptake of the DNA into the cells, the tubes were placed on ice for 30 min, heat shocked for 20 s at 42°C in a water bath and placed back on ice for a further 2 min. LB Broth Lennox (Invitrogen) (see Appendix B) was pre-warmed to 37°C and 950 µl was added to each transformation tube. The tubes were incubated at 37°C, with shaking (at ~250 rpm) for 1 h (Minitron Incubator Shaker, Infors HT). The contents of the tubes were then plated as described below.

2.5.2.2 Plating *E. coli* cells transformed with pEGFP-N1 and glycerol stocks of *E. coli* cells transformed with pN1-V1bR onto selective plates

To produce colonies to seed the cultures with, the transformed *E. coli* were first plated onto agar plates. Immediately following the transformation procedure described in Section 2.5.2.1, the transformed *E. coli* were plated onto selective plates (see Appendix B) containing ampicillin (100 µg/ml) (see Appendix B for stock solution detail) and kanamycin (30 µg/ml) for pUC19 and pEGFP-N1 transformed cells respectively. The untransformed *E. coli* were also plated onto plates containing kanamycin. To ensure separate colonies could be identified and picked to seed the starter cultures, three different volumes of the suspension of pEGFP-N1 transformed bacteria (20 µl, 100 µl, and 200 µl), were plated onto separate plates. Aliquots of 100 µl of the appropriate bacterial suspensions were plated onto the untransformed negative control and the pUC19 positive control plates. The aliquoted volume was spread across the plates using a glass spreader. The plates were incubated overnight at 37°C. The

next morning these plates were examined for colonies, wrapped with plastic wrap followed by aluminium foil and stored upside down at 4°C, until needed to seed the starter culture.

When plating the glycerol stocks of pN1-V1bR transformed bacteria onto agar plates containing kanamycin (30 μg/ml), a scrapping was taken from a frozen glycerol stock with a sterile inoculating loop and streaked in a zig zag motion onto the plates. The plates were incubated and examined as described above.

2.5.2.3 Culturing of *E. coli* cells transformed with pEGFP-N1 or pN1-V1bR

In order to obtain sufficient plasmid DNA, *E. coli* colonies transformed with the plasmids were picked and bulk cultures were produced. Seeding a bulk culture with a single *E. coli* colony results in a slow growth rate. This is because of the low number of cells. Therefore, a single colony is transferred to a smaller starter culture first and cells from this starter culture are then used to seed the bulk culture.

2.5.2.3.1 Starter culture

Aliquots of LB Broth Lennox (3 ml) containing kanamycin (30 μg/ml) were added to six McCartney bottles and pre-warmed to 37°C. Individual colonies of pEGFP-N1 transformed bacteria were selected from the culture plates using yellow pipette tips and used to inoculate three of these McCartney bottles. The remaining three bottles were inoculated with individual colonies of pN1-V1bR transformed bacteria. The inoculated McCartney bottles were incubated at 37°C with shaking (at ~300 rpm) for 8 h.

2.5.2.3.2 Bulk culture

A 200 μl aliquot of the cloudiest starter culture for pEGFP-N1 was transferred to 100 ml of pre-warmed LB Broth Lennox containing kanamycin (30 μg/ml) in a 500 ml conical flask. A second 500 ml flask was similarly inoculated with pN1-V1bR. The flasks were incubated overnight at 37°C with shaking (at ~300 rpm). If the plasmid DNA could not be harvested immediately from the bulk cultures, the bulk cultures were stored at 4°C until harvesting could begin. Prior to harvesting the plasmid DNA, a 1 ml aliquot of each bulk culture was taken to make glycerol stocks of pEGFP-N1 and pN1-V1bR.

2.5.2.4 Plasmid purification

In order to transfect mammalian cells, plasmid DNA needs to be extremely pure. Therefore, it is important to use a plasmid purification procedure which ensures that high quality DNA is

produced. Here the Invitrogen PureLink™ HiPure Plasmid Purification Kit was used to harvest and purify the plasmid DNA, via the steps described below.

Before the harvesting and purification of the DNA was started, the RNase A was added to the Resuspension Buffer (R3), and the Lysis Buffer (L7) was checked to ensure no precipitate had formed. If a precipitate had formed it had to be re-suspended before use. The HiPure Filter Maxi Column, with the Filtration Cartridge inserted, was placed on a rack. Equilibration Buffer (EQ1) (30 ml) was added to the Columns and was allowed to drain by gravity flow. The addition of EQ1 ensures that the anion exchange resin in the columns is positively charged so that it will be able to bind the negatively charged DNA backbones when the cell lysates were added. While the EQ1 was dripping through the Columns, the cell lysates were prepared. The overnight bulk cultures were centrifuged (Sorvall® RC-6™ Plus, Thermo Scientific) at 4,000 x g for 10 min. The supernatants were discarded and 10 ml of R3 was added to each pellet. The cells were re-suspended in this buffer by re-pipetting until the suspension was homogeneous. This buffer contains RNase which breaks down any RNA released from within the cells once they have been lysed. The cell suspensions were transferred to individual 50 ml centrifuge tubes and 10 ml of L7 was added to each tube to lyse the cells. The solutions were mixed by gently inverting the tubes five times. This ensured an even concentration and distribution of the reagents for cellular breakdown. The resulting suspensions were incubated at room temperature for 5 min, to ensure the lysis of all cells. Precipitation Buffer (N3) (10 ml) was added to each cell lysate and immediately mixed by inversion of the tubes. N3 precipitates impurities in the lysate, clarifying it. It is important to mix these solutions gently by inversion as mixing with a vortex can result in shearing of the genomic DNA, leading to contamination of the plasmid DNA. Each lysate, including the precipitate was transferred to one of the prepared Columns and allowed to drain through by gravity flow, until there was less than 1 drop per 10 seconds. To increase the DNA yield the Columns were washed with 10 ml of Wash Buffer (W8), which was allowed to drain through by gravity flow. The flow through from the initial addition of the precipitated lysates to the Columns and the wash were discarded as waste along with the inner Filtration Cartridges. Each column was then washed with 50 ml of W8, which was allowed to drain through by gravity flow. Washing of the Columns under moderate salt conditions allows the DNA to stay bound to the Columns while removing the RNA, proteins, carbohydrates, and other impurities from the resin. The flow through from this wash was discarded as waste. Sterile centrifuge tubes were placed under the Columns and 15 ml of Elution Buffer was added to each column

to produce high salt conditions under which the plasmid DNA was eluted from the resin. The Columns were discarded at this point and the eluted plasmid DNA desalted and concentrated by alcohol precipitation. To do this, 10.5 ml of isopropanol was added to each eluate in the centrifuge tubes and mixed well. The tubes were centrifuged at 15,000 x g for 30 min at 4°C in a pre-cooled centrifuge (5810R, Eppendorf). The supernatants were carefully removed and discarded and 5 ml of 70% ethanol was added to re-suspend each pellet. The tubes were centrifuged at 15,000 x g for 5 min. These re-suspension and centrifugation steps were repeated once more. The pellets were then air dried for 10 min, and re-suspended in 500 µl of TE Buffer (TE). The resulting plasmid DNA solutions were transferred to microfuge tubes. The final concentration of DNA was determined using a spectrophotometer (ND1000, Nanodrop).

2.5.2.5 Screening of harvested pEGFP-N1 and pN1-V1bR plasmid DNA

To determine whether the plasmids amplified, harvested, and purified in the bulk preparation were indeed the desired plasmids, pEGFP-N1 and pN1-V1bR, the purified products were run on a DNA gel. The gel separates bands based on size, thus allowing determination of the product through size comparison with a DNA ladder containing bands of a known size.

The plasmids must be cut with a restriction enzyme before they are run on the gel as circular DNA migrates through gels at different rates to linear DNA (Sambrook, 1989). XhoI is a one cutter restriction enzyme for both plasmids. Therefore, prior to running the gel, restriction enzyme digests were made for both pEGFP-N1 and pN1-V1bR in 1.7 ml microfuge tubes (see Table 2.5 for volumes of the reagents). The reaction digest was incubated overnight at 37°C.

pEGFP-N1	pN1-V1bR
1 µl pEGFP-N1 (1 µg/µl)	1 µl pN1-V1bR (1 µg/µl)
2 µl 10x REact 2 Buffer	2 µl 10x REact 2 Buffer
2 µl XhoI (10 u/µl)	2 µl XhoI (10 u/µl)
15 µl Nanopure H_2O	15 µl Nanopure H_2O

Table 2.5 Restriction enzyme digests of pEGFP-N1 and pN1-V1bR. The table shows the composition of the restriction enzyme digests. Both digests were made up to a total volume of 20 µl. XhoI is from Invitrogen. Details of REact 2 Buffer can be found in Appendix B.

A 50 ml 0.8% agarose gel was made in 0.5x TBE, and 5 µl of SYBR® Safe DNA Gel Stain was added. The gel was poured (Easy cast, Owl Scientific) and the ten well comb inserted. A

High Mass DNA Ladder (Invitrogen), and uncut and cut plasmid solutions were made up with loading buffer (see Appendix B) as shown in Table 2.6 below. The gel was loaded with the samples and run at 80 V for ~40 min in 0.5x TBE running buffer. The gel was viewed under UV illumination (Multi Wave UV Transilluminator, UltraLum) to determine the position and size of the DNA bands. An image of the gel was taken under UV illumination on the SynGene ChemiGenius2 camera using Gene Snap software.

Lane #	DNA Solution	Volume of DNA Solution	Volume of ddH_2O	Gel Loading Dye
Lane 1	-	-	-	-
Lane 2	-	-	-	-
Lane 3	Ladder	4µl	-	1µl
Lane 4	pN1-V1bR Cut	6µl (RED)	4µl	2µl
Lane 5	pN1-V1bR Uncut	0.3µl (1µg/µl)	9.7µl	2µl
Lane 6	pEGFP-N1 Cut	6µl (RED)	4µl	2µl
Lane 7	pEGFP-N1 Uncut	0.3µl (1µg/µl)	9.7µl	2µl
Lane 8	Ladder	4µl	-	1µl
Lane 9	-	-	-	-
Lane 10	-	-	-	-

Table 2.6 Composition of solutions added to the gel. There is a total of 0.3 µg of DNA per lane. Note RED = restriction enzyme digest

2.6 OPTIMISATION OF ANTIBIOTIC SELECTION CONDITIONS

2.6.1 DETERMINATION OF THE G418 CONCENTRATION REQUIRED TO KILL ALL UNTRANSFECTED HEK293 CELLS

A range of eight different G418 concentrations were tested in triplicate over a period of 21 days using (untransfected) HEK293 cells plated into a 24 well plate. The percentage confluence and morphology of the HEK293 cells in the individual wells were assessed at 24 h intervals as a way to measure the effect of G418.

The HEK293 cells were plated into a 24 well plate at 2 x 10^4 cells/well in 1 ml of MEM+. After 24 h, 500 µl of MEM+ containing either 0, 300, 600, 1,200, 1,500, 2,100, 3,000, or 4,500 µg/ml of G418 (see Appendix B for stock solution details) was added to three replicate

wells per concentration. The plate was then gently rocked to mix the solutions in the wells. This addition gave final G418 concentrations of 0, 100, 200, 400, 500, 700, 1,000, or 1,500 µg/ml, respectively. The final plate layout once the antibiotic had been added is shown in Table 2.7.

	1	2	3	4	5	6
A	0 µg/ml	0 µg/ml	0 µg/ml	100 µg/ml	100 µg/ml	100 µg/ml
B	200 µg/ml	200 µg/ml	200 µg/ml	400 µg/ml	400 µg/ml	400 µg/ml
C	500 µg/ml	500 µg/ml	500 µg/ml	700 µg/ml	700 µg/ml	700 µg/ml
D	1000 µg/ml	1000 µg/ml	1000 µg/ml	1500 µg/ml	1500 µg/ml	1500 µg/ml

Table 2.7 Layout of the 24 well plate for the determination of an appropriate antibiotic selection concentration. In the table the final concentrations of G418 are indicated. Each concentration was tested in triplicate. A total of eight different concentrations were tested.

The medium in the wells was changed every three days after the initial addition of G418. This ensured that there was fresh medium for cell growth and that the antibiotic was replenished before it had broken down. The old medium was removed via aspiration and 1 ml of fresh medium containing the appropriate concentration of G418 was added to each well. The confluences of the replicate wells were averaged and graphed.

It is important to note that adding G418 stock solution to tissue culture medium lowers the pH. The antibiotic containing medium was therefore left in a Biological Safety Cabinet-Class II for 30-60 min (time depended upon the G418 concentration) to equilibrate to the normal pH. It was then placed into a CO_2 incubator to bring the temperature up to 37°C, before addition to the wells. Another option is to have a buffering system within the culture medium or within the G418 stock solution (Ausubel et al., 1994; Mortensen and Kingston, 2009).

2.6.2 OPTIMAL PLATING DENSITY DETERMINATION

Once an appropriate concentration of antibiotic was determined, the HEK293 cell plating density was optimised. The plating density was optimised to ensure that the HEK293 cells reached 80% confluence before major cell death occurred.

The HEK293 cells were plated at four different densities 2 x 10^4, 4 x 10^4, 6 x 10^4, and 8 x 10^4 cells/well in 1 ml of MEM+. The cells were plated onto two 24 well plates. Six wells were plated at each density per plate. After 24 h, G418 was added to three replicate wells per

plating density per plate. The remaining wells were used as a control and therefore only had MEM+ added. A G418 concentration of 500 µg/ml was used for plate one and a G418 concentration of 700 µg/ml was used for plate two. Initially the G418 was introduced by adding 500 µl of 1500 µg/ml G418 in MEM+ to the specified wells on plate one, and 500 µl of 2100 µg/ml G418 in MEM+ to the specified wells on plate two. The plates were rocked gently to mix the solutions. Table 2.8 shows the plate layout common to both plates. The table also indicates which wells were used as test wells and which were used as control wells. Every three days after this initial addition of G418, the medium was removed from all the wells by aspiration, and then 1 ml of the appropriate solution was added back using a P1000 Gilson pipette. The plates were cultured for a total of 21 days. The wells were assessed at 24 h intervals for percentage confluence and morphology. The confluence values of the replicate wells within each treatment group were averaged and graphed.

	1	2	3	4	5	6
A	2×10^4 cells/well T	2×10^4 cells/well T	2×10^4 cells/well T	2×10^4 cells/well C	2×10^4 cells/well C	2×10^4 cells/well C
B	4×10^4 cells/well T	4×10^4 cells/well T	4×10^4 cells/well T	4×10^4 cells/well C	4×10^4 cells/well C	4×10^4 cells/well C
C	6×10^4 cells/well T	6×10^4 cells/well T	6×10^4 cells/well T	6×10^4 cells/well C	6×10^4 cells/well C	6×10^4 cells/well C
D	8×10^4 cells/well T	8×10^4 cells/well T	8×10^4 cells/well T	8×10^4 cells/well C	8×10^4 cells/well C	8×10^4 cells/well C

Table 2.8 Layout of the 24 well plates for the determination of an optimal plating density. The plate layout in the table indicates the plating density for each well. T indicates that the well was a test well and it was therefore treated with antibiotic (500 µg/ml for plate one, 700 µg/ml for plate two). C indicates that the well was a control well and it was therefore not treated with antibiotic**.**

It was discovered that during the above experiment the CO_2 incubator was operating at a CO_2 concentration below 5%. This potentially affected HEK293 cell growth. Therefore, this experiment was partially repeated with the following changes:

1) HEK293 cells were plated at only two densities (6×10^4 and 8×10^4 cells/well in 1ml of MEM+) into one 24 well plate.
2) The cells in this experiment were cultured for only seven days.

The two different concentrations of G418 were then added to three wells at each plating density as in the previous experiment. The percentage confluence of the cells in each well and the cell morphology were determined, recorded, and analysed as previously described.

2.6.3 DATA ANALYSIS

The data were collected from these experiments as confluences and morphologies for individual wells. The data from the replicate wells (n=3) were entered into GraphPad Prism 4. Prism was used to calculate the means ± SEM and graph the results as a line graph. The line graphs produced can be seen in Section 3.3. These graphs were used to determine which concentrations of G418 were successful in killing all of the (untransfected) HEK293 cells. They were also used to determine the appropriate plating density.

2.7 TRANSFECTION OF HEK293 CELLS USING F6

The F6 transfection protocol using HEK293 cells was optimised following the manufacturers guidelines (Roche Applied Science). The finalised protocol is described below.

HEK293 cells were plated into a 24 well plate at 60,000 cells/well in 0.5 ml MEM+. The four sides of the plate were tapped once each to ensure an even distribution of the cells across well. These cells were then cultured for 24 h to allow time for the cells to adhere to the base of the well. This culture time also allowed for cell growth. It is important to ensure that the cells are between 50-80% confluent when they are transfected, as this is the optimal confluence range for transfection and ensures that the cells are in their log phase of growth.

In order to have the transfection complex ready at a specified time point, preparation was started ~1 h in advance. The complex was prepared by mixing F6(µl) with DNA(µg) at a 3:1 ratio in medium free from serum (MEM-). In a 24 well plate, depending on the DNA concentration, ~20 µl of the complex was added to each of the wells, which contained cells plus 0.5 ml MEM+. A master mix of complex was prepared to ensure that all wells received exactly the same mixture of transfection complex.

The steps outlined below were used to determine the volumes required of the three solutions required to prepare the master mix. To allow for pipetting error the volume made up was 10% greater than the minimum amount required for the number of wells to be transfected.

1) Number of wells = n
2) Volume of MEM-/well = 19.4 µl
3) Volume of F6/well = 0.6 µl
4) Amount of DNA/well = 0.2 µg

Therefore if the DNA concentration = x µg/µl, the volume of DNA/well = (0.2 µg / x) µl

Therefore the volumes of the complex components per well = 19.4 µl MEM- + 0.6 µl F6 + (0.2 µg/ x) µl DNA

Therefore total volume of master mix = (n + (n/10)) (19.4 µl MEM- + 0.6 µl F6 + (0.2 µg/ x) µl DNA)

The transfection complex was made in a laminar flow hood (CF43S, Gelman Sciences), in clearly labelled, sterile, 1.7 ml microfuge tubes. The MEM- was aliquoted first. The F6 was then removed from the refrigerator ~15 min prior to use to allow it to warm to room temperature before the vial was opened. It was mixed by tapping the vial and then vortexing it for 1 s to deposit it at the bottom of the vial. Once mixed, the F6 was added directly to the MEM in the microfuge tube. It was very important at this step that the F6 did not come into contact with the sides of the tube, as contact with plastic adversely affects transfection efficiency. The tube was tapped to mix the F6 and MEM and then centrifuged for 1-2 s to bring the solution to the bottom of the tube. The solution was incubated for 5 min at room temperature, and the plasmid DNA was then added. The tube was tapped to mix the solutions then centrifuged for 1-2 s. The resulting solution was incubated at room temperature for 30 min to allow the complex to form. At 30 min the appropriate volume of the complex solution was added to each well, containing cells to be transfected, on the 24 well plate, in a drop wise manner, using a P20 Gilson pipette. The pipette tip was changed between the individual wells. After the complex had been added to the wells, the plate was swirled gently to ensure an even distribution of the complex. The cells were then incubated in a CO_2 incubator for a minimum of 48 h before being used in experiments. This incubation ensured sufficient time for gene expression.

2.8 STABLE TRANSFECTION

2.8.1 PLATING AND TRANSFECTION OF HEK293 CELLS WITH pEGFP-N1 AND pN1-V1bR PLASMID DNA

HEK293 cells (untransfected) were plated into the wells of three six-well plates, at 250,000 cells/well in 2 ml of MEM+. The plates were tapped once on each side to ensure an even distribution of the cells. The cells were then incubated for 24 h, at which time they were transfected with pEGFP-N1 or pN1-V1bR. The transfection complex was made according to the protocol in Section 2.7. The volumes in the transfection formula were adjusted to accommodate a six well plate. The adjusted volumes per well were:

97 µl MEM- + 3 µl F6 + 1 µg DNA

The cells in well number one were left as an untransfected negative control and the cells in well number two were transfected with pEGFP-N1 as a positive control. All of the cells in the remaining sixteen wells were transfected with pN1-V1bR.

2.8.2 SELECTION AND ISOLATION OF CLONAL CELLS STABLY-EXPRESSING pEGFP-N1 AND pN1-V1bR USING G418

In order to establish clonal cell lines stably-expressing the transfected plasmid, clonal lines must be selected for and then isolated. After selection and isolated, the lines can then be assessed and used for further research.

After the transfection, the cells were incubated for 48 h, at which time they had reached ~90% confluence. At 48 h each of the individual wells were re-plated into 10 cm plates. This re-plating was achieved by, removing the medium from the wells of one plate then adding 1 ml of T/E to each well. The plate was swirled so that the T/E covered all of the cells. The T/E was then removed and the plate incubated for 8 min at room temperature. After the incubation, 1 ml of MEM+ was added to each of the wells, while the plate was tilted on an angle. This dislodged the cells from the base of the well. The resulting cell suspension was re-pipetted eleven times with a P1000 Gilson pipette set to 0.8 ml. After re-pipetting, all of the suspension from one well was transferred into the corresponding 10 cm plate. The plate contained 11 ml of pre-warmed MEM+. The cells in the 10 cm plates were incubated for 5-6 h to give the cells time to adhere. After this incubation, 4 ml of MEM+ containing 2000 µg/ml G418 was added to each plate. This produced a final G418 concentration of

500 μg/ml. The medium in the plates was replaced every three days. The replacement MEM+ contained 500 μg/ml G418. The cells were cultured in these plates for two weeks.

After two weeks, all of the untransfected control cells were non-viable and the transfected cells had reached over 90% confluence. Ten of the 10 cm plates (one with cells transfected with pEGFP-N1 and nine with cells transfected with pN1-V1bR) were re-plated into new 10 cm plates at a plating density of 1.25 x 10^4 cells/plate. The plate numbers were reduced as all seventeen of the plates contained cells that had been successfully transfected. The cells were re-plated at a low density to ensure that the colonies picked were clonal. In order to re-plate the ten plates in a timely fashion one plate was trypsinised and the cells counted (see Section 2.3.3 for cell counting). As all the plates were approximately the same confluence this cell count was assumed to be a good estimate of cell numbers for all of the plates. This cell count was therefore used to calculate the required dilution for all of the plates. The re-plating was achieved by, removing the medium from the plate and adding 4 ml of T/E. The plate was swirled to ensure that the T/E had covered all of the cells and the T/E was then swiftly removed. The plate was incubated for 8 min at room temperature. Then 6 ml of MEM+ without G418 was added to the plate. The resulting cell suspension was re-pipetted eleven times using a 5 ml plastic pipette. A diluted aliquot of each cell suspension was then added to the appropriate new 10 cm plate, which contained 15 ml of pre-warmed MEM+ with 500 μg/ml G418. The morning after the re-plating, all of the plates were examined for any signs of unusual cell morphology as an indicator of cell viability, and to ensure that the cells had adhered as single cells. The cells were cultured for approximately one week in these plates, which allowed time for colony growth. The MEM+ with 500 μg/ml G418 was replenished every three days.

To select individual colonies from the 10 cm plates the medium was removed and the plates were washed once with 3 ml of sterile 1x PBS (see Appendix B). The plates were then air dried for ~3 min. During this time three colonies (that were about the size of a pin head) were selected and their position marked on the base of the plate. After air drying, 15 μl of 1 x PBS was added to an individual colony using a P20 Gilson pipette. The colony was scraped with the pipette tip to remove it from the base of the plate. The cells were re-pipetted, 4-6 times to form a suspension. The cell suspension was then removed to an individual well of a 24 well plate, which contained 1 ml of MEM+ with 500 μg/ml G418. Each of the ten 10 cm plates had three individual colonies removed. The cells in the 24 well plates were cultured until one well, of the three containing cells picked from the same 10 cm plate, had reached over 50%

confluence. There were two exceptions to this, in which two wells seeded with colonies from the same 10 cm plate were continued further. This was done because the cells from these 10 cm plates had morphologies closest to that of the parental (untransfected) HEK293 cell line. During culture of the cells in the 24 well plates the MEM+ with 500 μg/ml G418 was changed every three days.

Once one of the three wells had reached over 50% confluence the cells from that well were re-plated back into a 10 cm plate at 1.25 x 10^4 cells/plate. The process of colony selection was then repeated as described above. Colony selection was repeated to ensure the cell lines established were clonal. To re-plate from a well in a 24 cm plate back into a 10 cm plate, the medium was removed from the well and 0.2 ml of T/E was added. The plate was swirled to cover all of the cells with the T/E. The T/E was then swiftly removed and the plate incubated at 37°C in a CO_2 incubator for 5 min. This incubation is a departure from the normal protocol of trypsinisation (Section 2.3.2). The T/E incubation step was changed to minimise adverse effects on the remaining cells in the 24 well plate, which were not re-plated at the same time. Following the 5 min incubation, 0.4 ml of MEM+ with 500 μg/ml of G418 was added to the well. The cell suspension was re-pipetted, with a P1000 Gilson pipette set to 0.32 ml, eleven times. The cells were counted (see Section 2.3.3), then diluted and added to the new 10 cm plate.

2.8.3 PRODUCTION OF STABLY TRANSFECTED CLONAL CELL LINE STOCKS

Once the cells had been through the colony selection and isolation process a second time the cells were transferred to a 25 cm^2 flask for the production of clonal cell line stocks. To do this, the first well of the three to reach over 50% confluence was removed from the 24 well plate as described above except, all of the cells that were not used for counting were transferred to a 25 cm^2 flask. Once the cells were removed to the 25 cm^2 flask they were named and identified as an individual stably-transfected clonal cell line. Once the clonal cell lines had reached over 50% confluence in the 25 cm^2 flasks, they were sub-cultured into separate 75 cm^2 flasks (see Section 2.3.2). Once the 75 cm^2 flask reach ~80% confluence the cells were frozen for storage (see Section 2.3.4). The frozen cell stocks were then used to assess the stably-transfected clonal cell lines.

2.9 IP ASSAY

2.9.1 PLATING AND TRANSFECTION

HEK293 cells, either untransfected or stably-transfected were plated into wells of Primaria™ 24-well Multiwell™ plates (Becton Dickson). The cells were plated at 60,000 cells/well in either 0.5 ml (untransfected) or 1 ml (stably-transfected) MEM+. For each condition to be tested in the assay, three replicate wells were plated. The cells were then incubated for 24 h. Wells containing untransfected HEK293 cells were transiently transfected at 24 h (see Section 2.7). Wells containing stably-transfected cells were only assessed for percentage confluence and cell morphology at 24 h. Approximately 22 h after the transient transfection another 0.5 ml of MEM+ was added to these wells. This addition ensured that there were sufficient nutrients for cell growth. Nothing was added to the wells containing the stably-transfected HEK293 cells at this time point. After this point the untransfected and stably-transfected cells were treated in the same manner.

2.9.2 LABELLING WITH *myo*-[^{3}H]inositol

The cells were radio-labelled 52 h after plating. To do this, the medium was removed from the wells and they were washed once with 0.8 ml Dulbecco's Modified Eagle Medium (DMEM) without inositol (MP Biomedical). The wells were then radio-labelled with 0.5 ml of labelling medium (see Appendix B). The cells were incubated in this medium for 20 h. This ensured that the *myo*-[^{3}H]inositol (Perkin Elmer Inc.) was incorporated into PIP_2 via inositol metabolism by the cells.

2.9.3 TREATMENT OF CELLS WITH AVP

After the cells were labelled with *myo*-[^{3}H]inositol, they were assessed for IP production in response to AVP stimulation as an indicator of functional expression of the V1bR. Two protocols were used for this

1) measurement of the total IP production in response to stimulation with AVP (100 nM, 15 min),
2) measurement of IP production in response to stimulation with AVP (100 nM, 15 min) with or without an AVP (5 nM, 5 min) pre-treatment, as an indicator of receptor desensitisation to AVP.

These protocols differed in their wash and AVP-stimulation regimes. The protocols for the separation of inositol phosphate metabolites and the counting of samples were the same.

2.9.3.1 Wash and AVP stimulation of HEK293 cells transfected with pN1-V1bR

HEK293 cells have low levels of adherence and therefore have a tendency to lift from the base of the well in cell-based assays, as these assays consist of a number of washes within quick succession. Due to this tendency to lift, cultureware coatings promoting cell adhesion and careful technique were required to remove and add the washes.

2.9.3.1.1 Determination of responsiveness to a 100 nM, 15 min stimulation with AVP

The plate was removed from the incubator after labelling of the cells and all of the wells were washed once with 0.5 ml of pre-warmed "basal solution" (see Appendix B). Half of the wells were designated as "basal" wells, and these received another 0.5 ml of the "basal solution" (0 nM AVP). The remaining wells were stimulated with AVP, and these received 0.5 ml of pre-warmed "stimulation solution" (100 nM AVP) (see Appendix B). The plate was then incubated at 37°C for 15 min, in a CO_2 incubator.

2.9.3.1.2 Determination of AVP responsiveness and magnitude of desensitisation following an AVP pre-treatment

The plate was removed from the incubator after labelling and all of the wells were washed once with 0.5 ml of the "control solution" (see Appendix B). The cells in half of the wells were pre-treated with AVP and these received 0.5 ml of pre-warmed "pre-treatment solution" (5 nM AVP) (see Appendix B). The other half of the wells were not pre-treated with AVP and these received 0.5 ml of pre-warmed "control solution" (0 nM AVP). The plate was then incubated in a CO_2 incubator at 37°C for 5 min. After 5 min, the plate was removed from the incubator and all of the wells washed twice with 0.5 ml of "basal solution". The cells in half of the control wells and half of the pre-treated wells were not stimulated with AVP and these received 0.5 ml of the "basal solution" (0 nM AVP). The cells in the remaining wells were stimulated with AVP and these received 0.5 ml of the "stimulation solution" (100 nM AVP). The plate was then incubated at 37°C for 15 min, in a CO_2 incubator.

2.9.4 SEPARATION OF INOSITOL PHOSPHATE METABOLITES BY ANION EXCHANGE CHROMATOGRAPHY

Polyprep Chromatography Columns (Biorad Laboratories) were set up in a rack over a waste collection tray, and 2 ml of resin (Biorad AG1-X8), mixed with Nanopure H_2O, at a 1 g:1 ml ratio, was added to each. Once the excess water had dripped through a resin bed of approximately 1.6 ml remained.

After the 15 min test incubation described above, the plate was immediately removed from the incubator and placed on ice where 0.5 ml of ice cold "stop" solution (see Appendix B) was added to each well. The plate was left on ice and 1 min later 0.5 ml of "neutralisation" solution (see Appendix B) was added to each well. The cells in each well were then lysed and transferred to their respective columns. To do this, the base of each well was scraped with a P1000 Gilson pipette tip to dislodge the cells. The cell suspension was then vigorously re-pipetted seven times to lyse the cells and transferred to the corresponding column. All solutions that were added to the columns were added slowly and evenly with the tip against the side of the column just above the solution level, to minimise resin disturbance. The pipette tip was changed between each well, to avoid cross-contamination.

Solutions that were added to the columns were allowed to drip through before the next solution was added. After the contents of all of the wells had been added to the columns, the columns were washed with 10 ml of Nanopure H_2O to elute free inositol. The columns were then rinsed with 8 ml of elution buffer II (see Appendix B) in order to elute glycerophosphoinositides. After elution buffer II had finished dripping through all of the columns, the ends of the columns were blotted with paper towels to remove any remaining drops. Labelled collection vials were then placed underneath the columns. To elute the inositol phosphates, 3 ml of elution buffer VI (see Appendix B) was added to the columns and the eluates were collected into the vials. Once elution buffer VI had dripped through, the ends of the columns were blotted and the collection vials were removed. If the columns were to be used again on the same day, this is the point at which they were regenerated. To regenerate the columns they were first rinsed with 10 ml of regeneration solution (see Appendix B), which removed any remaining inositol phosphate metabolites. The columns were then washed three times with 10 ml of Nanopure H_2O. This re-equilibrated the columns so that they were ready for the next batch of samples.

2.9.5 COUNTING OF THE SAMPLES

The levels of [^{3}H]-IPs in the eluates produced from the IP assay were measured by liquid scintillation counting. One ml of each eluate was transferred to a scintillation vial, to which 10 ml of the scintillation cocktail (OPTIPHASE 'HISAFE' 3, Perkin Elmer Inc.) was added. The scintillation vials were then capped and shaken well to mix the two solutions. This produced a solution with a cloudy appearance. To allow this to clear before counting, the vials were left for ~20 min in the dark. Once clear, each scintillation vial was transferred to the liquid scintillation counter (Wallac 1410, Perkin Elmer). The sides of the vials were not

touched to avoid the potential for counting errors produced by smears. The samples were counted for 20 min each using the "CPM count" function. This counts per minute (CPM) function is used when all of the samples are expected to have similar counting efficiencies. The counter will determine the CPM by fitting a reference spectrum to the spectrum of the unknown sample. The software will then calculates the CPM value that will produce the spectrum based on stored information. The readout produced by the counter contains the values for the following parameters for each sample: CPM (corrected counts per minute), SQPI (spectral quench parameter for ^{3}H), and CPM1 (uncorrected CPM). The SQPI (sample quenching) was typically between 105-113 for all samples. The counter produced the corrected CPM value by performing background and chemiluminescence corrections to the raw counts. The corrected CPM values of replicate sample were averaged and the SEM calculated by hand.

2.9.6 ANALYSIS OF THE ASSAY RESULTS

The IP response of cells to agonist stimulation in this research was expressed as the CPM or the stimulated response as a percentage of the control response, as has been done previously (Freedman et al., 1997; Gaudreau et al., 2002). The CPM values of replicate wells in individual experiments were averaged and the SEM calculated to produce a mean CPM value for a specific treatment within an experiment. The CPM mean values from replicate experiments were entered into GraphPad Prism 4, which was used to calculate the overall mean ± SEM, and the results were plotted in a bar graph. The stimulated response data was calculated by expressing the stimulated response with or without a pre-treatment as a percentage of the control response with or without a pre-treatment. The calculated values of percentage stimulation with or with out an AVP pre-treatment were then entered into Prism, which was used to calculate the overall mean ± SEM, and the results were then plotted in a bar graph. The significance of differences in the IP responses to AVP stimulation was determined through *t*-tests conducted using Prism software. A result of $P<0.05$ was considered to be significant. The actual levels of significance are as indicated in the text. All data were reported as mean ± SEM.

All graphs of IP assay results were created using GraphPad Prism 4. The Prism data analysis outputs were used to created tables using Microsoft Excel 2007.

2.10 WASTE DISPOSAL

Waste contaminated with either the HEK293 cells or *E. coli* DH5 alpha cells is bio-hazardous and was disposed of as follows.

2.10.1 DISPOSAL OF HEK293 CELL WASTE

Liquid waste containing HEK293 cells was treated with 1% Virkon overnight, prior to being flushed down the sink with running water. Any plasticware used for HEK293 cell work was autoclaved prior to disposal with the normal rubbish. Any glassware that came in contact with HEK293 cells was treated with 1% Virkon overnight. The Virkon was then flushed down the sink with running water, and the glassware was washed as normal.

2.10.2 DISPOSAL OF *E. COLI* DH5 ALPHA WASTE

Liquid contaminated with the DH5 alpha *E. coli* was collected in a waste container and autoclave before being flushed down the sink with running water. The only exception to this was any waste that also contained alcohol. This was collected separately and treated with 1% Virkon overnight before being flushed down the sink with running water. Any plastic ware that came into contact with the DH5 alpha *E. coli* was autoclaved before being disposed of with the normal rubbish. Any glassware to come in contact with DH5 alpha *E. coli* was treated with 1% Virkon overnight. The Virkon was then flushed down the sink with running water, and the glassware was washed as normal

2.10.3 RADIOACTIVE WASTE DISPOSAL

Waste from the IP assays was mixed waste containing both bio-hazardous HEK293 cells and radioisotope (^{3}H). The waste was first treated with 1% Virkon overnight and then flushed down the sink with running water for a minimum of 30 min. All of the plasticware that came into contact with HEK293 cells was first treated overnight with 1% Virkon. The plasticware and rubbish was then collected together and disposed of in rubbish bags according to National Radiation Laboratory requirements. During the IP assay, no glassware came into contact with the isotope.

CHAPTER 3:

PREPARATION FOR AND GENERATION OF V1BR-STABLY TRANSFECTED HEK293 CELL LINES

3.1 MEASUREMENT OF HEK293 CELL PROLIFERATION RATE

3.1.1 INTRODUCTION

Cell line growth follows a characteristic progression (Freshney, 2000). It begins with the lag phase, where little to no cell growth is seen. Then there is the exponential growth phase, which is also known as the log phase. During this phase there is rapid linear cell growth. Finally there is the stationary phase, which is also known as the plateau phase. During this phase again there is little to no cell growth. The results of cell experiments can be affected by their growth phase. Therefore, when transfecting cells with F6 (see Section 2.7) the manufacturer (Roche Applied Science) recommends for good quality, consistent transfection results that the cells are transfected during their log phase of growth, preferably when the cells are between 50-80% confluent. The antibiotic G418, used in this research to select stably transfected cells, also works best against log phase cells (Southern and Berg, 1982).

Plasmid DNA complexed with carrier molecule transfection reagents is believed to enter the cell by utilising the endocytosis pathway (Grimm, 2004). However, the DNA must still reach the nucleus, especially for the production of stable transfectants. This happens most efficiently when the cells are dividing as the nuclear envelope ruptures and the DNA gains access to the host genome (reviewed in Hebert, 2003; Vaughan et al., 2006). G418 works best against rapidly dividing cells as it interferes with ribosomal function and therefore translation (Southern and Berg, 1982), which occurs at the highest rate when cells are rapidly dividing. Cell cultures are utilised as model systems as they are generally homogenous populations of cells (Freshney, 2000). However, it must be taken into account that the cells are generally most uniform when they are in the log phase of growth as opposed to the plateau phase. Therefore, ideally all experiments should be conducted while the cells are in log phase. To achieve this goal careful planning is required.

As the growth phases of the cells can have a significant impact on experimental outcomes it is important to have information on the growth characteristics of a particular cell line. This

growth information allows informed decisions to be made during the planning stages of experiments and this planning promotes successful experimental outcomes. To obtain growth characteristic information on HEK293 cells, a growth curve was constructed for this (untransfected) cell line. This growth curve was then used to determine the phases of growth for HEK293 cells. The protocol developed for the measurement of cell proliferation rate (see Section 2.4) was optimised from that described by Freshney (2000), and used to obtain the growth curves shown below.

3.1.2 RESULTS

The growth curves for the two different plating densities are shown in Figure 3.1. These curves were obtained in collaboration with Katherine van Bysterveldt, a fellow MSc student. The growth curves developed for cells plated at both of the densities showed a classic sigmoid shape. There was a lag phase of 2 days, which was then followed by a linear growth of 4-6 days. These curves have the same linear slope, and the cell numbers appear to be doubling approximately every 24 h. The supplier of the HEK293 cells, ATCC, lists HEK293 cells as having a doubling time of 34 h. The doubling time measured here was about one third less than this. After the linear growth phase both curves level off indicating a plateau phase.

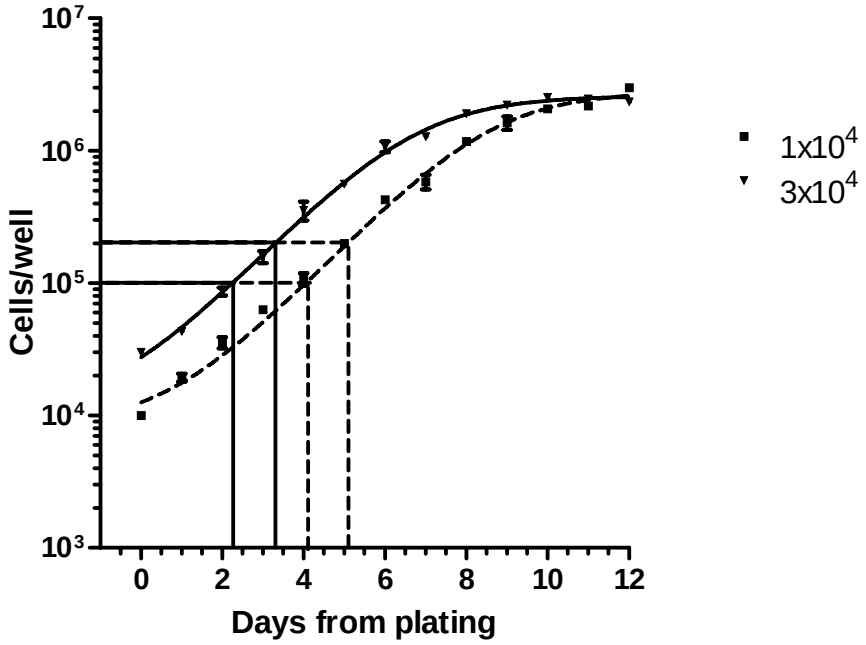

Figure 3.1 Growth curves of HEK293 cells. Cells were either plated at $1x10^4$ or $3x10^4$ cells/well in a 24 well plate. Growth was then measured over 10-12 days. The curves have sigmoid appearances, which indicate the three phases of growth, lag, log, and plateau. Both curves have the same linear slope, and appear to double in number approximately every 24 h during this phase. This was determined by the time taken to increase cell numbers from 1×10^5 to 2×10^5. The data is expressed as mean ± SEM first 10 days (n=2), last 2 days (n=1).

Each day in addition to counting the cells, the average percentage confluence was determined for all wells plated at the same density. This allowed the phases of cell growth to be correlated with percentage confluences (Table 3.1). From the table it can be seen that between 20-90% confluence cells are within their log phase of growth and hence suitable for use in experiments. This information enabled future assessment of the cells using an inverted microscope to provide information not only on the confluence of the cells but also on their growth phase. It is an ideal range as cells between 50-80% confluent are seen to be in the log phase of growth, which is optimal for transfection. It also confirms that HEK293 cells should be sub-cultured when they are between 50-80% confluent, in order to maintain a uniform cell culture.

Growth Phase	Approx. % Confluence
Lag	< 20%
Log	20-90%
Plateau	> 90%

Table 3.1 The percentage confluences correlating to the different growth phases of HEK293 cells.

3.2 PREPARATION OF PLASMID DNA FOR TRANSFECTION

3.2.1 SUB-CLONING OF THE rV1bR GENE INTO A SUITABLE VECTOR (pEGFP-N1) FOR STABLE TRANSFECTION

3.2.1.1 Introduction

Vector design is an important step to ensuring the successful delivery of plasmid DNA into cells, especially when using non-viral techniques (Mairhofer and Grabherr, 2008). In order to get the best possible results when stably transfecting mammalian cells, it is preferable to have the GOI on the same plasmid as the selection marker. An alternative possibility is to co-transfect the cells with two different plasmids. However, by transfecting with only one plasmid there is increased confidence that if the selection marker is expressed the GOI will be also. This means that fewer stable clones will require screening in order to find the ones which are expressing the GOI.

Previously in this laboratory, pALTER®-MAX (Figure 2.2) and prV1bR plasmids, obtained by Dr. Drusilla Mason as a gift from Dr. Greti Aguilera (National Institutes of Health (NIH), Bethesda, USA) were used to transiently transfect HEK293 cells (Gatehouse, 2008). The full rV1bR clone (~2.6 kb) (Lolait et al., 1995) was inserted into pALTER®-MAX (5.5 kb) to produced prV1bR (~8.1 kb). pALTER®-Max was originally from Promega and the prV1bR plasmid was made by Bioserve – Biotechnologies (MD, USA). However, the prV1bR plasmid alone is unsuitable for stable transfections as it does not contain a gene for mammalian selection. The pEGFP-N1 vector (Figure 2.3), obtained as a gift from Dr. Gabriele Dachs (University of Otago, Christchurch), contains the *neo* gene for mammalian selection. pEGFP-N1 (4.7 kb) was originally obtained from Clontech.

It was decided to sub-clone the GOI, rV1bR, into the pEGFP-N1 plasmid rather than use a co-transfection method to create the stable cell lines. The sub-cloned plasmid was created using the protocol described in Section 2.5.1. It was decided to remove EGFP (~0.7 kb) in this process for two reasons:

1) to keep the plasmid size as small as possible to increase transfection efficiency, and
2) to reduce the potential for the production of artefacts in V1bR signalling by attaching EGFP to the end of rV1bR.

The fact that the *neo* gene provides for selection in both prokaryotic and eukaryotic cells (Southern and Berg, 1982) makes it particularly useful, as it halves the number of selection markers required. This helps to keep the plasmid size as small as possible, and hence improves transfection efficiency (Mairhofer and Grabherr, 2008).

3.2.1.2 Results

The success of the sub-cloning of the rV1bR gene into the pEGFP-N1 with the removal of the EGFP gene is illustrated in the image (Figure 3.2) of the gel that was run at the end of the sub-cloning protocol (see Section 2.5.1.6). The four lanes directly to the right of the ladder, containing the restriction enzyme digested plasmids from mini preparations #1 - #4 had clear separation of the bands. The bands were of the appropriate size to be the destination vector (pN1 ~4.0 kb) and the rV1bR gene (~2.6 kb). Due to the more intense light signal from mini preparation #1, two bands are not obvious in the image, but were clearly separate when the gel was viewed directly. This data indicates that these four plasmid DNA preparations contain the desired plasmid construct. The mini preparations #5 through 10 did not show clear separation of the bands, and thus it is unclear whether these preparations do contain the

desired construct or just the destination vector. It is possible that two bands could not be seen due to DNA overloading. It is also possible that some of the *E. coli* colonies that grew were only transformed with the destination vector as this contained the selection marker.

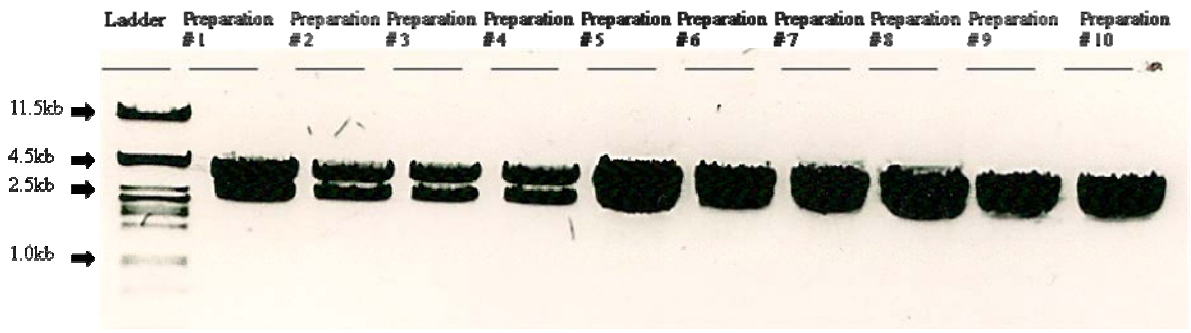

Figure 3.2 Image of the gel on which the digested sub-cloned plasmid DNA preparations were run. The four preparations run immediately to the right of the ladder show separation of the bands indicating the presence of both the destination vector pN1 (~ 4.0 kb) and the GOI rV1bR (~2.6 kb). The lane immediately to the right of the ladder does not show clear band separation in the image but did when the gel was viewed directly. The other six preparations did not show clear separation. This may have been due to excess DNA in the lanes.

Preparation #1 was selected for use in further research as it appears to have the highest plasmid DNA concentration of the four preparations. In Table 3.2 results from spectrophotometry analysis of this preparation can be seen. The overnight culture, which preparation #1 was made from, was used to create glycerol stocks so that larger preparations of plasmid DNA could be made in the future. All other preparations and cultures were discarded. This new construct, designated pN1-V1bR is ~6.6kb in size. The size of the plasmid has increased as the *egfp* gene (~0.7 kb) that was removed is much smaller in size than the rV1bR (~2.6 kb).

	Concentration	260/280	260/230
pN1-V1bR	309.4 ng/µl	1.90	2.12

Table 3.2 Spectrophotometer results for plasmid preparation #1. A 260/280 ratio of above 1.8 is considered pure for DNA. The 260/230 ratios are a secondary measure of purity, these are usually higher than the 260/280 ratio, typically between 1.8 – 2.2. These data show that the construct purity was acceptable and the preparation was suitable for mammalian transfections.

3.2.2 BULK PREPARATION OF PLASMID DNA

3.2.2.1 Introduction

For the transfection optimisation (using pEGFP-N1), the IP assay modification (using pN1-V1bR), and the stable transfection (using pN1-V1bR), large amounts of highly purified plasmid DNA was required. pEGFP-N1 rather then pN1-V1bR was used for the optimisation of the transfection protocol for HEK293 cells with F6, because the presence of a fluorescent gene in the vector simplified the assessment of transfection efficiency. In order to have enough plasmid DNA stock for all these experiments large plasmid DNA preparations were required of both plasmids. To make plasmid DNA preparations, competent bacterial cells are transformed with the plasmids and cultured under antibiotic selection conditions. The plasmid DNA can then be harvested from the bacterial cell cultures and purified to the high quality required for mammalian cell transfection.

It is important that the plasmid DNA used to transfect mammalian cells is highly purified, as contaminants such cellular DNA, RNA, and protein, and endotoxins produced by bacteria can affect the transfection efficiency. The extracted DNA can be assessed for cellular contaminates using spectrophotometry. The presence of endotoxins are more difficult to detect, but can be reduced by using a protocol specifically designed to exclude endotoxins. Effects of endotoxins are apparent when levels are greater than 2000 endotoxin units (EU)/ µg DNA, with cell proliferation and viability affected above 10,000 EU/ug DNA (Butash et al., 2000). Sufficient reduction in endotoxin levels can usually be achieved through purifying the DNA using anion exchange resin, although endotoxin free conditions may be needed for certain *in vivo* studies (Butash et al., 2000). To achieve good results from mammalian transfections, plasmid DNA with the greatest possible purity is preferred. High quality DNA was ensured by using the Invitrogen PureLink™ HiPure Plasmid Purification Kit to harvest and purify the plasmid DNA as described in Section 2.5.2.

Once a plasmid DNA preparation has been made it is important to show that it is of the correct plasmid and if there is any contamination. To do this a DNA gel was run and the bands examined. As the two plasmids purified here are different sizes, the gel also enabled determination of whether cross contamination had occurred. Circular DNA migrates through a gel at a different rate to linear DNA (Sambrook, 1989). This difference depends on a number of properties of the gel. To compare circular plasmid DNA with the linear DNA ladder to determine its size, the plasmid must linearised with a restriction enzyme. It is important that this restriction enzyme will only cut the plasmid once, as this ensures that the plasmid runs as

a single linear DNA fragment and that the band produced will be representative of the actual plasmid size. To determine whether the same restriction enzyme could be used for both plasmids the list of restriction enzymes sites for pEGFP-N1 was examined for an enzyme that would cut at only one site. This site needed to be in the part of the vector that was present in both plasmids. There is only one XhoI site in pEGFP-N1, and this is in the appropriate portion of the plasmid. XhoI has been used previously in this laboratory as a one-cutter for prV1bR (Gatehouse, 2008), and it was determined at that time that there are no sites for XhoI in the rV1bR sequence. The XhoI site in prV1bR plasmid is outside the region that was transferred when the rV1bR was sub-cloned into pN1. XhoI was therefore selected as an appropriate restriction enzyme for cutting the two plasmids prior to running them on a gel.

3.2.2.2 Results

The maxi plasmid DNA preparations produced 500 µl of DNA for each plasmid. Both of these products were assessed using the spectrophotometer. Table 3.3 shows the concentration and purity of the prepared plasmids. This table shows that large quantities of both plasmids were produced, with a high level of purity. Before running the gel described in Section 2.5.2.5, the plasmids were digested with the restriction enzyme XhoI. The restriction enzyme digest of each was run alongside the uncut plasmids as well as a DNA ladder. An image of the resulting gel taken under UV illumination can be seen in Figure 3.3. This shows both plasmids were successfully prepared, as they are present in the appropriate size regions of the gel. This result combined with the data from the spectrophotometer shows that the plasmids produced are what were expected and have been purified to a standard suitable for use in mammalian transfections. This means that the experiments that required the large amounts of high pure plasmid DNA could proceed.

	pN1-V1bR	pEGFP-N1
260/280	1.89	1.90
260/230	2.28	2.27
Concentration	1643.8 ng/µl	1819.2 ng/µl

Table 3.3 Plasmid DNA preparation purities and concentrations. A 260/280 ratio of above 1.8 is considered pure for DNA. The 260/230 ratios are a secondary measure of purity, these are usually higher than the 260/280 ratio, typically between 1.8 – 2.2. The spectrophotometer results above show that the plasmid preparations were successfully purified.

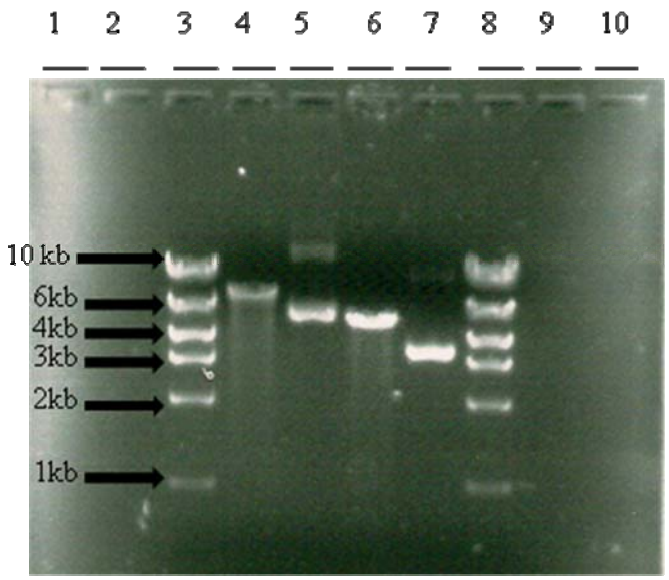

Figure 3.3 Image of the gel run to confirm plasmid identity. Lanes 3 and 8 show the ladder, lane 4 shows pN1-V1bR cut with XhoI, lane 5 shows pN1-V1bR uncut, lane 6 shows pEGFP-N1 cut with XhoI, and lane 7 shows pEGFP-N1 uncut. The ladder band sizes are indicated on the side. This shows that the cut pN1-V1bR is of a slightly greater weight than 6 kb, corresponding to its size of ~6.6 kb. The cut pEGFP-N1 is approximately mid-way between 4 kb and 6 kb corresponding to its size of 4.7 kb.

3.3 OPTIMISATION OF ANTIBIOTIC SELECTION CONDITIONS

3.3.1 INTRODUCTION

Approximately one in 10^4 transfected cells will stably integrate the plasmid DNA (Ausubel et al., 1994; Haber, 1999; Mortensen and Kingston, 2009). Therefore, a selection marker is required in order isolate these cells from the ones in which transfection was unsuccessful or was only of a transient nature. Prior to use of the selection marker in selecting stably transfected cells, appropriate selection conditions must be determined through testing the marker against the (untransfected) parental cell line. In this case selection conditions were determined using (untransfected) HEK293 cells.

The construct used, for selection of stably expressing clonal cell lines, contains the selection gene *neo* (see Section 3.2). Therefore, appropriate selection conditions needed to be determined for G418 (Invitrogen) with parental (untransfected) HEK293 cells. The concentration of G418 required to kill a specific cell lines varies and therefore a wide range of concentrations were tested (Mortensen and Kingston, 2009; Southern and Berg, 1982). As different lots of the antibiotic can have different potencies, each lot used in this research was

tested as well. However, lot testing was done over a much smaller range of G418 concentrations, and one pre-determined plating density.

Determination of the selection conditions consisted of two parts:

1) determination of the concentration of G418 sufficient to kill all parental (untransfected) HEK293 cells, and
2) determination of an optimal plating density for the selection process, using this G418 concentration.

To chose the range of G418 concentrations to use for determining an appropriate selection concentration, a number of sources were consulted including Southern and Berg (1982), Ausubel (1994), Violin (2008), Mortensen and Kingston (2009), and the Ambion p*Silencer neo* Expression Vector instructions. The plating densities chosen were a range of those regularly used in this laboratory when plating HEK293 cells.

There are two key time points in determining the appropriate antibiotic selection conditions:

1) massive cell death within 7-9 days, and
2) complete cell death within 2-3 weeks.

Ideally the cells should reach ~80% confluence before they start to die. This can be adjusted through optimisation of the plating density. A maintenance concentration of the selection marker is also required to ensure the cells maintain expression when passaged normally after the original selection process. This concentration is commonly half that required for the original selection of the stable transfectants.

The protocols used to optimise the antibiotic selection conditions (see Section 2.6) were adapted from the Ambion p*Silencer neo* Expression Vector instructions, Invitrogen pcDNA3.1(+) pcDNA3.2(-) instructions, and from the protocols reported by Ausubel (1994).

3.3.2 RESULTS

3.3.2.1 Selection and maintenance concentrations of G418

Eight different concentrations of G418 were tested in the range of 0 μg/ml to 1,500 μg/ml. The results were graphed and can be seen in Figure 3.4. This figure shows that a G418 concentration of 100 μg/ml had minimal effect on the growth of HEK293 cells, only reducing confluence by 10-15% when compared with the control concentration of 0 μg/ml. Concentrations of G418 between 200 μg/ml and 1,500 μg/ml were able to reduce cell

numbers to varying degrees, as indicated by a drop in confluence. These concentrations killed some of the cells as indicated by a change in cell morphology from a normal neuronal morphology (Figure 3.5a) to small round structures with a grainy appearance (Figure 3.5b). The viability of cells with the latter morphology was assessed through trypan blue exclusion. All cells with this changed morphology took up the stain showing they were no longer viable. Concentrations of G418 of 500 µg/ml and above were all successful in killing all of the HEK293 cells within 2-3 weeks.

In this experiment the HEK293 cells did not reach ~80 % confluence before they started to die, suggesting that the plating density needed to be optimised. Two concentrations of G418 (500 µg/ml and 700 µg/ml) were chosen to for this optimisation experiment. These concentrations were chosen as they were the lowest and second to lowest concentrations that caused a large amount of death after 7-9 days and killed all of the cells within 3 weeks. Using two concentrations rather then one meant a greater range of options could be assessed.

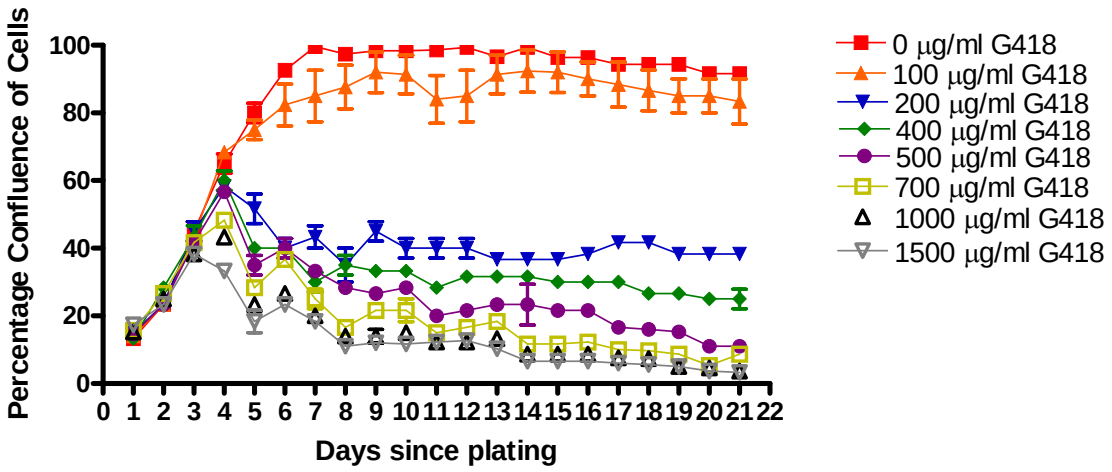

Figure 3.4 Determination of an appropriate antibiotic selection concentration. Graph of percentage confluence of untransfected HEK293 cells treated with varying concentrations of G418. All cells in the experiment were plated at 2 x 10^4 cells/well in a 24 well plate. The data shown are mean ± SEM (n=3)

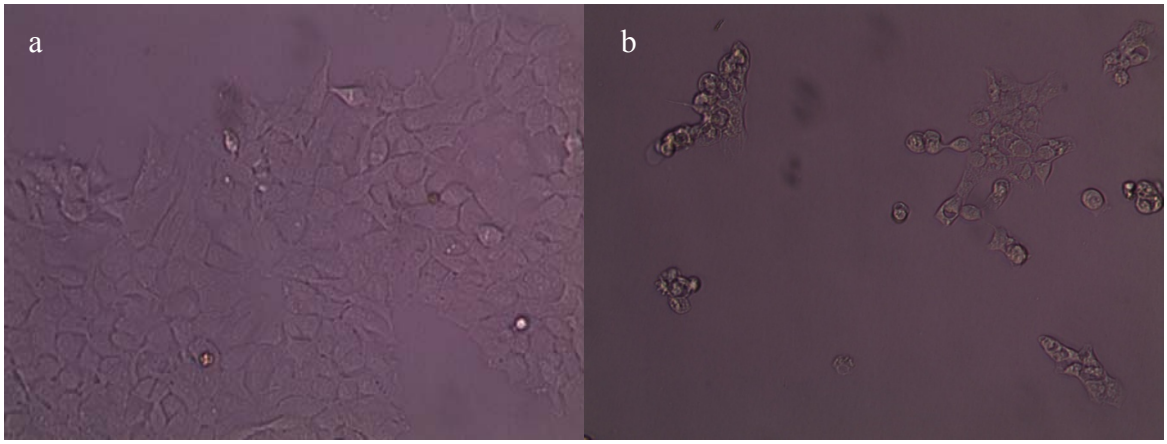

Figure 3.5 Images of HEK293 cells treated with 0 µg/ml and 1,500 µg/ml G418. Both images were taken on day 4 of the experiment. a) Shows HEK293 cells treated with 0 µg/ml G418. The cells still look like normal HEK293 cells, neuronal in morphology and growing in a monolayer. b) Shows HEK293 cells treated with 1,500 µg/ml G418. The cells have become circular in morphology, with a grainy appearance and the cellular monolayer can be seen to be breaking up.

3.3.2.2 Optimal plating density for antibiotic selection

Four different plating densities were assessed, with the two G418 concentrations selected above: 2 x 10^4, 4 x 10^4, 6 x 10^4, and 8 x 10^4 cell/well in a 24 well plate. The results of this experiment can be seen in Figures 3.6 (plate one - 500 µg/ml) and Figure 3.7 (plate two - 700 µg/ml). These figures show that all of the G418 treated cells were effectively killed within 2-3 weeks. This occurred slightly faster at the G418 concentration of 700 µg/ml. Only two of the initial plating densities, 6 x 10^4 and 8 x 10^4 cell/well, reached close to 80% confluence before the cells began to die.

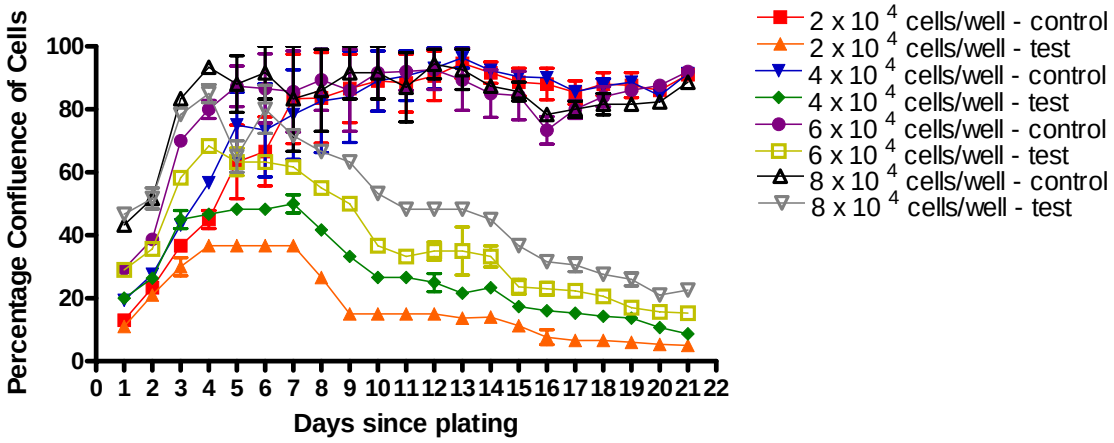

Figure 3.6 Determination of the optimal plating density with 500 µg/ml G418. 0 µg/ml and 500 µg/ml G418 were tested with different initial plating densities of HEK293 cells in a 24 well plate. The test wells received the G418. The data are expressed as percentage confluences, mean ± SEM (n=3).

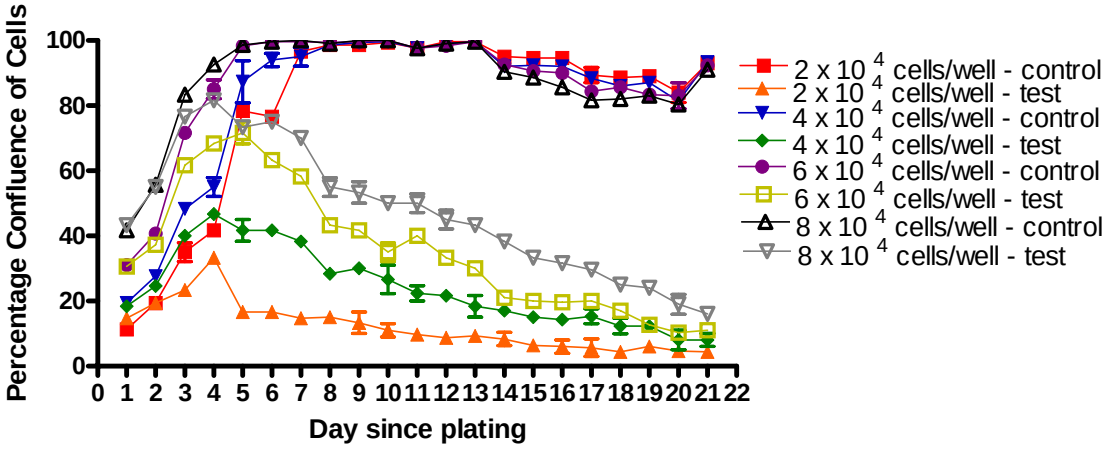

Figure 3.7 Determination of the optimal plating density with 700 µg/ml G418. 0 µg/ml and 700 µg/ml G418 were used with different initial plating densities of HEK293 cells in a 24 well plate. The test wells received the G418. The data are expressed as percentage confluences, mean ± SEM (n=3).

After this experiment it was discovered that the CO_2 incubator had not been maintaining the interior environment at 5% CO_2, 95% air. This may have negatively impacted the growth of the HEK293 cells, because the correct CO_2 level is required to maintain the normal pH of the culture medium. Therefore, the first seven days of the experiment were repeated with both G418 concentrations. However, only the two higher initial plating densities (6 x 10^4 and 8 x 10^4 cells/well) were used. The results from this second experiment can be seen in Figure 3.8. This shows that the cells at both of the initial plating densities reached ~80% confluence before beginning to die, at both of the G418 concentrations.

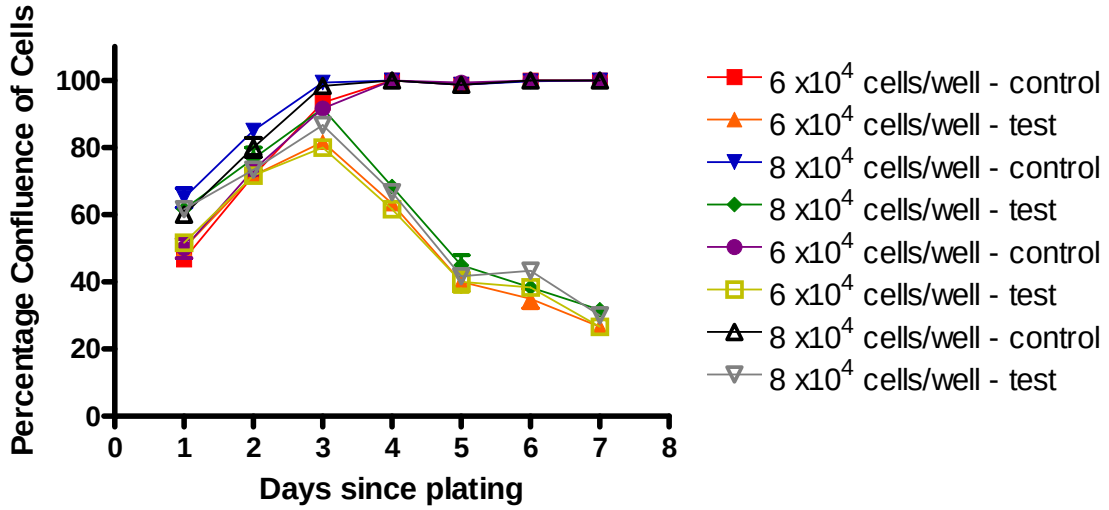

Figure 3.8 Second determination of optimal plating density. 0 µg/ml, 500 µg/ml, and 700 µg/ml G418 were used at the initial plating densities of 6 x 10^4 and 8 x 10^4 cells/well in a 24 well plate. Test wells were treated with G418. Data obtained with cells treated with 500 µg/ml are shown in orange and dark green, and those obtained with 700 µg/ml are shown in light green and grey. The experiment was conducted over 7 days. Data are expressed as percentage confluences, mean ± SEM (n=3).

The information from these experiments allowed the determination of the minimum concentration of G418 required to kill the untransfected parental cell line. This was 500 µg/ml G418. Therefore, the maintenance concentration was set at 250 µg/ml G418. These experiments showed that plating densities equivalent to 6 x 10^4 cells/well and above in a 24 well plate or equivalent were appropriate.

3.4 OPTIMISATION OF F6 TRANSFECTION PROTOCOL

3.4.1 INTRODUCTION

There are many methods which can be used to transfect mammalian cells with plasmid DNA including carrier molecules, viruses, and electroporation (Bonetta, 2005; Mortensen and Kingston, 2009). An advantage of carrier molecules over other methods is that these protocols are generally simple and do not require expensive equipment (Bonetta, 2005). It is important when generating stable cell lines to ensure high levels of transfection, as the more DNA that is introduced to a cell the better the chance of it being stably integrated (Mortensen and Kingston, 2009). Maintaining a high level of functional viability is also an important concern. Many lipid based transfection reagents including non-liposomal reagents provide ease of use and low level of cytotoxicity (Bonetta, 2005), along with high levels of transfection. Previous

work in this laboratory used HEK293 cells that were transiently transfected using Lipofectamine™ 2000 (Invitrogen) (Gatehouse, 2008). A disadvantage with using this transfection reagent is that it must be used with cells at a high confluence to minimise cytotoxicity (see manufacturers product insert). This is a disadvantage when trying to ensure log phase cells for subsequent experiments. It was noted that some research groups were using F6 to transfect HEK293 cells to produce stable lines and Lipofectamine™ 2000 to transfect other cell lines, such as U2-OS cells (Violin et al., 2006) and mouse embryonic kidney cells (Violin et al., 2008). Correspondence with the authors confirmed that they had found F6 resulted in lower levels of HEK293 cell cytotoxicity than Lipofectamine™ 2000. F6 has been shown to produce high levels of transfection and low levels of cytotoxicity across a wide range of cell lines (Jacobsen et al., 2004). Therefore, the decision was made to switch transfection reagents in order to minimise cytotoxicity during transfection and maximise the likelihood of success in generating clonal stable cell lines.

Transfection results can be improved for a specific cell line by optimising a number of the parameters. The efficiency of a transfection reagent depends mostly upon the cell line used (Sambrook, 1989), rather than the plasmid it is transfected with. Therefore, when optimising F6 for use with HEK293 cells, a variety of plasmids can be used. Optimising the protocol utilising transient transfections will generally produce a protocol with conditions suitable for producing stable transfectants (Mortensen and Kingston, 2009). Therefore, transient transfections were used for optimisation.

Three parameters were evaluated during optimisation of the transfection protocol. These were selected based on information from the manufacturer (Roche Applied Sciences), and from Bonetta (2005) and Jacobsen et al. (2004) who examined transfection reagents and F6 optimisation respectively. The parameters evaluated were:

1) the ratio of F6 (µl) to DNA (µg),
2) the time required for complex formation, and
3) the time required for the cells to express the transfected gene

In order to determine the optimal ratio and times for the above parameters, an efficient method of analysis was required. Therefore, the HEK293 cells were transiently transfected with pEGFP-N1. Expression of the *egfp* gene produces a fluorescent protein, EGFP (excitation maximum = 488 nm, emission maximum = 507 nm), which can be evaluated using

confocal microscopy (TCS SP5, Leica). As the cells are assessed by viewing them, the same cells can be used to assess different time points. In contrast, optimisation using pN1-V1bR and a functional assay to assess V1bR expression, would be time consuming and the cells in a particular well could only be assessed at one time point. The final version of the transfection protocol can be seen in Section 2.7. Only the three parameters described above were altered; every other step was carried out as described in the final protocol. The experiment was repeated three times with some modifications (see below).

3.4.2 RESULTS

The data from the experiments assessing the three transfection parameters were collated into a single graph for ease of comparison (Figure 3.12). Confocal microscopy images of the transiently transfected cells (for an example see Figure 3.13), were used to calculate the percentage of cells fluorescing at each time point. Percentage fluorescence was used as a measure of the percentage of cells transfected as it was assumed that only the cells expressing EGFP had been transfected. To produce the data for the 24 h time point, five images were taken for each well in conserved positions of the well: top, upper middle, middle, lower middle and base. The percentage fluorescence was calculated for each of the five images. These percentages were used to produce an average percentage fluorescence for each well, which represented a specific combination of conditions. The equivalent wells in each of three replicate experiments were then averaged and the SEM calculated. At 48 and 72 h only one image was taken for each well. This was conserved at the upper middle position as it was generally the most representative of the images for a well. Two of the replicate experiments were taken to the 48 h time point. The wells in the two replicates treated with the same conditions were averaged and the SEM calculated. Only one of the replicate experiments was taken out to72 h. Therefore, the percentage fluorescence for this time point is based on only one count and therefore no SEM could be calculated.

3.4.2.1 Ratio of F6 (µl) to DNA (µg)

The first parameter examined was the ratio of F6 (µl) to DNA (µg). Three different F6:DNA ratios were assessed, 3:1, 3:2, and 6:1. All three were trialled first in an initial pilot experiment (data not shown). In this experiment, a sharp drop off in the transfection efficiency was seen for cells that were transfected at the 3:2 ratio, when compared to cells transfected with either of the other ratios. Therefore, the 3:2 ratio was excluded from further optimisation experiments in order to simplify them.

Figure 3.9 shows the percentage fluorescence 24 h post transfection. This figure shows that the F6:DNA ratio of 3:1 has consistently higher levels of transfection efficiency than the 6:1 ratio, when the two other parameters were held constant. This consistency made the decision to use the 3:1 ratio for transfection clear.

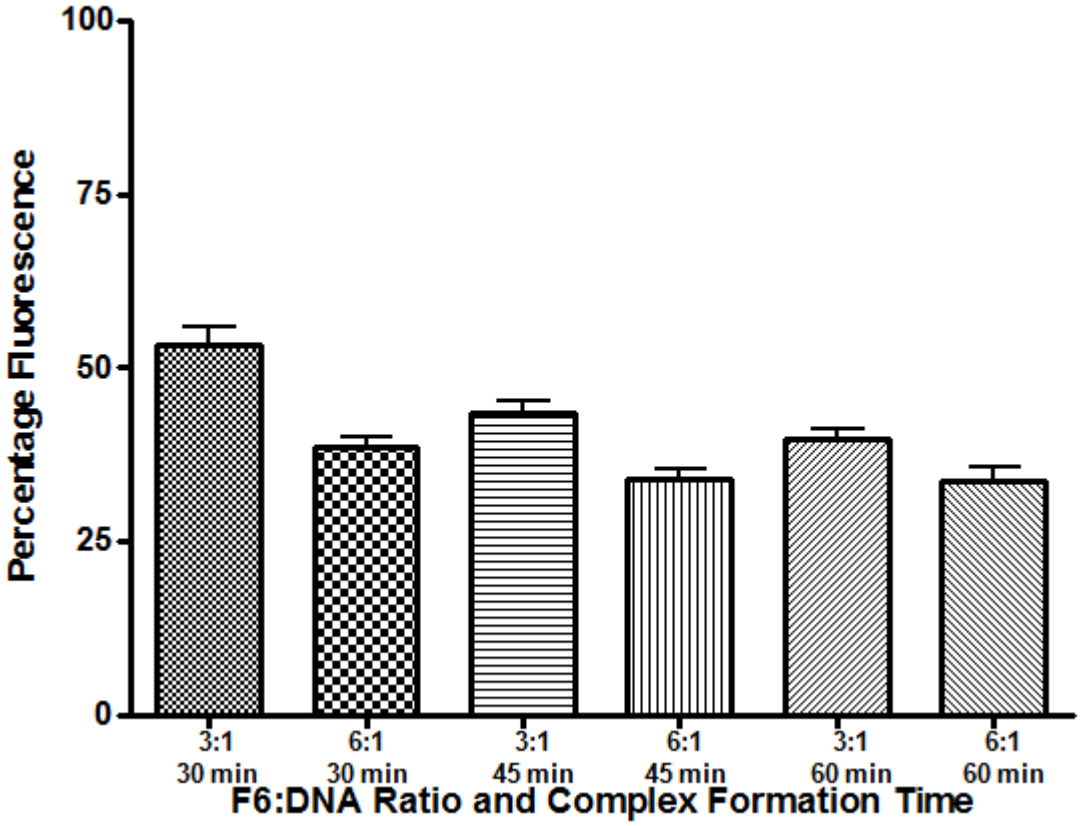

Figure 3.9 Percentage fluorescence when treated with different F6:DNA ratios and complex formation times. The data were determined from images taken 24 h after transfection. It can be seen the F6:DNA ratio of 3:1 produces a greater level of fluorescence at all complex formation times assessed. Data are mean ± SEM (n=3).

3.4.2.2 Time required for complex formation between F6 and DNA

The second parameter examined was the length of time for complex formation. Four different complex formation times were examined, 15, 30, 45, and 60 min. The complex must have a minimum of 15 min to form. Reducing this time will reduce the transfection efficiency (Jacobsen et al., 2004). However, it has been shown that the transfection efficiency can be increased with HEK293 cells when the complex formation time is increased. An initial pilot

experiment was run that included complex formation times of 15, 30, and 45 min (data not shown). The transfection efficiency was low with a complex formation time of 15 min when compared with efficiencies with either of the other complex formation times. Therefore, it was decided to exclude this complex formation time from the final optimisation experiments and instead include the longer complex formation time of 60 min (Figure 3.10). A complex formation time of 30 min has a greater level of transfection efficiency then any of the other complex formation times assessed. Therefore, 30 min was chosen as the complex formation time for transfection experiments.

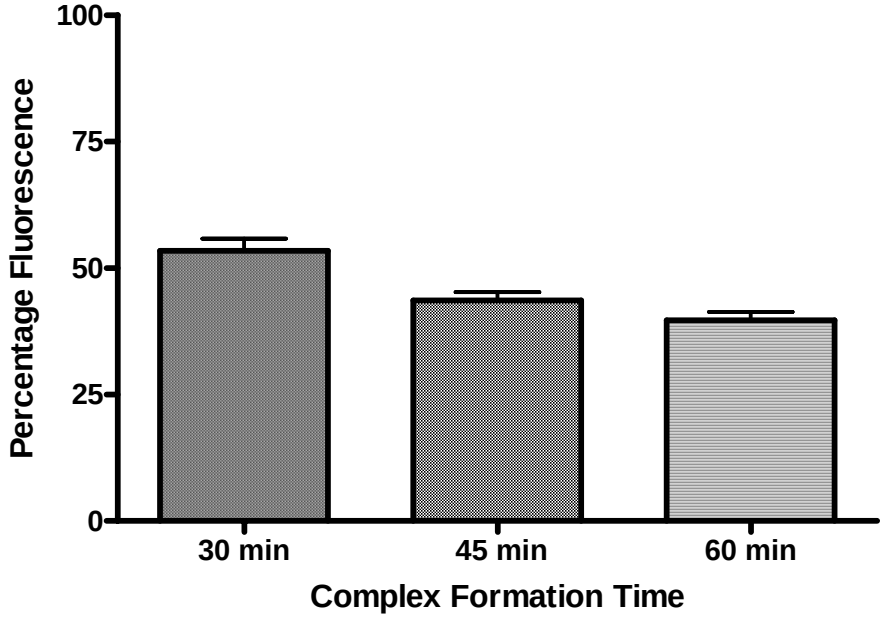

Figure 3.10 Complex formation time. The percentage fluorescence was determined from images taken at 24 h post transfection, with a F6:DNA ratio of 3:1. It can be seen that the complex formation time of 30 min produces the highest percentage fluorescence. Data are mean ± SEM.

3.4.2.3 Time required for expression of the transfected gene

The third parameter examined was the incubation time required for the HEK293 cells to take up the complex and express the gene. Three different incubation times were examined, 24 h, 48 h, and 72 h. As seen in Figure 3.11, percentage fluorescence increased between 24 and 48 h, but little difference was seen between 48 and 72 h. This indicates to a large increase in the number of transfected cell up to 48 h. Therefore, an incubation time of 48 h

was chosen as a suitable time for gene expression by the cells. An optimal EGFP expression time of 48 h has also been seen in other experiments using this plasmid (Hunt et al., 2010).

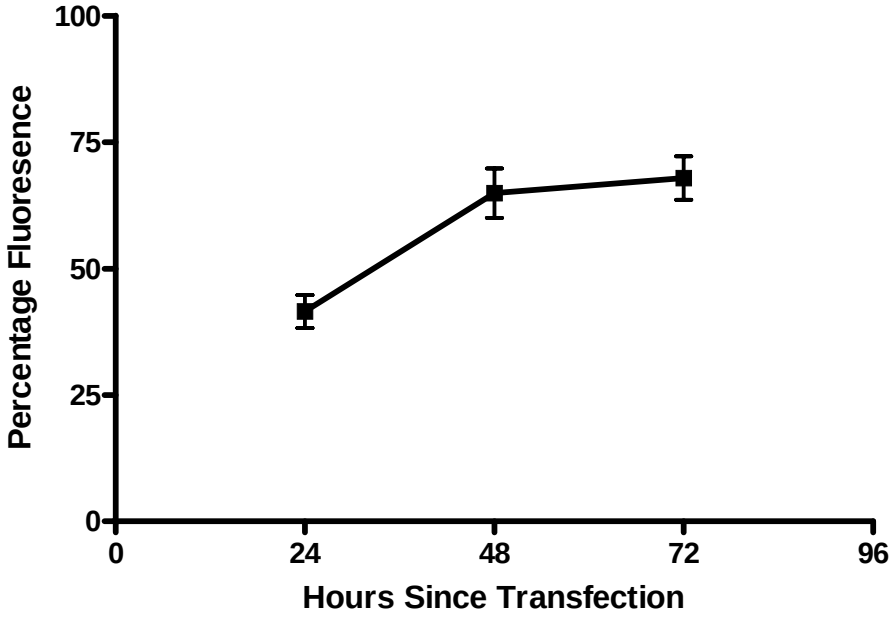

Figure 3.11 Percentage fluorescence over time. It can be seen that there is an large increase in percentage fluorescence between 24 and 48 h. However, between 48 and 72 h it plateaus. The data for each time point are the mean fluorescence of all the F6:DNA ratios and complex formation times assessed at that time point. The data are expressed as the mean ± SEM (n=6).

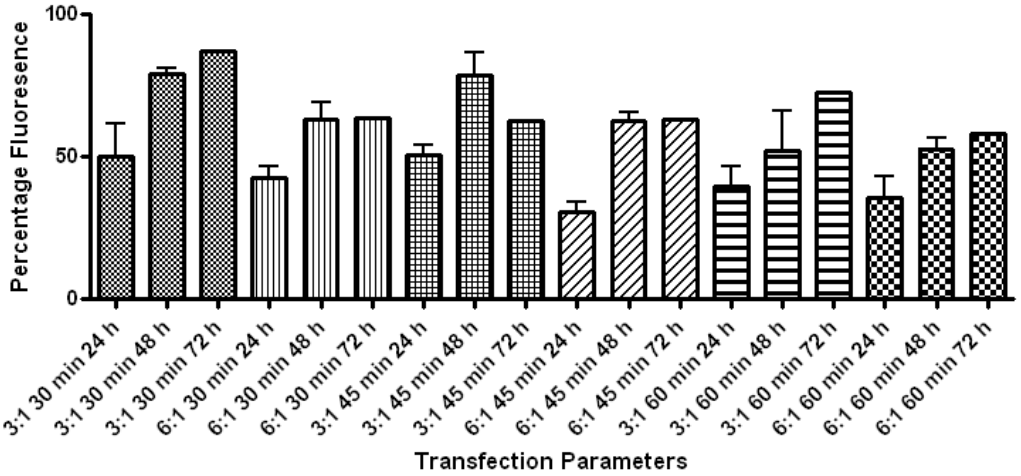

Figure 3.12 Optimisation of HEK293 cell transfection with F6. The data were collated from three replicate experiments, allowing for the best combination of conditions to be chosen: F6:DNA ratio (3:1), duration of complex formation (30 min) and time since transfection (48 h). The data are expressed as percentage fluorescence, mean ± SEM, 24 h (n=3), 48 h (n=2), 72 h (n=1).

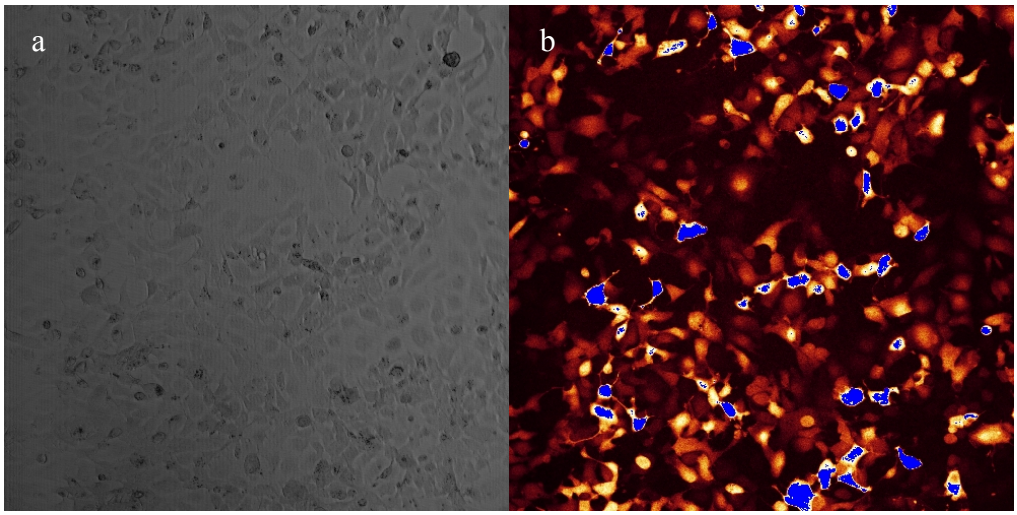

Figure 3.13 Confocal microscopy images of HEK293 cells expressing pEGFP-N1. a) The bright field image, and b) the fluorescent image are of cells 48 h post transfection with an F6:DNA ratio of 6:1 and a complex formation time of 45 min. These images are examples of those taken with the confocal microscope in order to determine the percentage fluorescence. Both images were taken at the same magnification and show the same field of view to allow for direct comparison

3.4.3 IMPACT OF THE TRANSFECTION PROTOCOL ON THE IP ASSAY PROTOCOL

The optimised transfected conditions affected the incubation times used for the IP assay. Previous use of the assay in this laboratory assessed expression of the rV1bR at 24 h post transfection (Gatehouse, 2008). Based on these transfection optimisation results, the optimal time to evaluate the rV1bR function was likely to be 48 h post transfection. This change in protocol increased the IP assay duration by one day. To maintain protocol consistency and therefore a consistent growth period, the increased time was conserved for both transiently and stably transfected cells.

3.5 GENERATION OF A MODEL CELL SYSTEM STABLY-TRANSFECTED WITH pN1-V1bR

3.5.1 INTRODUCTION

Stably-transfected model cell systems are a commonly utilised tool in the examination of gene expression and function (Mortensen and Kingston, 2009). Mammalian model cell system are also often utilised in the production of recombinant proteins to be used as pharmaceuticals (Wurm, 2004). This is because, unlike prokaryotic and plants cell systems, mammalian cells can perform the correct post-translational modifications on proteins for use in humans and other animals (Sambrook, 1989; Wurm, 2004).

In order to develop a stably-transfected model cell system, optimal for a specific research purpose, a significant amount of preliminary work and planning is required (Mortensen and Kingston, 2009). This ensures that the model cell system produced meets the requirements for the subsequent experiments, and therefore that reliable and meaningful data are produced. The preliminary steps completed in this research for the production of the model cell system are described in the previous sections of the Chapter. This section deals with the transfection and G418 selection of stably-transfected clonal cell lines. There were a few parameters within the stable transfection protocol (see Section 2.8), which were optimised to ensure a streamlined and successful completion. The protocol used to generate the stably-transfected cell lines was developed from information provided by Southern and Berg (1982), Roche Applied Science (see instructions for G418 use), Ausubel (1994) Mortensen and Kingston (2009), and discussions with Dr. Gabriele Dachs (University of Otago, Christchurch).

3.5.2 ASSESSMENT OF PROTOCOL

Cells were either left untransfected, or they were transfected with pEGFP-N1 or pN1-V1bR. The untransfected cells were used as a negative control for transfection. This was assessed when the G418 was added to the cells and all of the untransfected control cells died as they were not resistant to the antibiotic. The cells transfected with pEGFP-N1 were used as a positive control for transfection, because EGFP expression could be assessed by fluorescence confocal microscopy at any time point during generation of the clonal cell lines.

Two days after transfection the cells in each well of the six well plates were transferred to a 10 cm plate, given time to adhere, and then the G418 was added for the first time, to select for stable transfectants. Presumably because of the high DNA transfection rate of F6, there were too many stably-transfected cells transferred to each plate to allow identification of individual colonies after the death of the non-stably transfected cells. Therefore, the stably-transfected cells were re-plated at a much lower density into second 10 cm plate for colony selection. This extra step could be removed by reducing the density of transfected cells plated into the first of these two 10 cm plates, negating the need for replating after G418 addition. Based on confluence data, transferring approximately half of the cells in each well of the six well plates to each of the 10 cm plates should be a sufficient reduction. However, this appears to be lower than the optimised plating density (see Section 3.3.2.2) and requires confirmation before the protocol is adapted.

Two different methods of picking individual colonies from the 10 cm plates were tested;

1) The colony was removed, without removal of the medium, using a short glass Pasteur pipette with a flame-smoothened edge or a P200 Gilson pipette. Colonies were selected and picked while examining the plate under an inverted microscope, set up in a Biological Safety Cabinet-Class II. The colonies were scraped and the loosened cells sucked up with the pipette and then transferred to a well in a 24 well plate.
2) The details of the other method are as outlined in the protocol (see Section 2.8.2). Briefly, the medium was removed from the plate and the plate was washed with 1x PBS. The colonies of cells were then allowed to air dry. The chosen colonies were marked, and then removed by adding 15 µl 1x PBS directly to the colony, scraping it then removing the cell suspension.

The second method proved to be more reproducible than the first. With the first method the cells dispersed more easily in the medium and therefore the whole colony was not always transferred across to the 24 well plate. The glass pipette the tip tended to slide across the base of the plate with greater ease and, in doing so, sometimes more than one colony was scrapped off the plate surface. The second method put the cells under greater stress, as they were exposed to the air for 3-6 min. However, using this method was more straightforward, as colonies could be seen and picked without the microscope. This made it possible to ensure that only one colony was picked and that all the cells were transferred. Better results were therefore achieved when using this method.

Three colonies were picked off every 10 cm plate and transferred to individual wells of a 24 well plate. This was necessary as some of the transferred colonies did not grow in the 24 well plate. Other issues with some of the colonies included: very slow growth rates and changed morphologies. The cells from colonies picked from two of the 10 cm plates grew very well compared to others. This was a benefit as having more than one colony available allowed a greater number of colonies with normal morphologies and growth rates to be chosen.

After the second selection process the cells from the seed colonies were then established as individual clonal cell lines as indicated in Section 2.8.3. Once established as individual lines, they were named, for example clonal cell line 1C5-210210. The first number, in this case 1, indicates the number of the 24 well plate that the colony was transferred to in the second round of colony selection. The letter and the second number, in this case C5, indicate the well which the colony was transferred to. The suffix number, 210210, indicates the date when the cells were transfected, February 21st 2010. The suffix is therefore the same for all of the

clonal cell lines. Ten individual clonal cell lines were established. These were then screened and assessed as is described in Chapter 4.

3.6 MODIFICATION AND OPTIMISATION OF IP ASSAY PROTOCOL

3.6.1 INTRODUCTION

An assay of the levels of intracellular IPs produced in response to AVP stimulation of cells was used as an indicator of functional rV1bR expression (see Chapter 4). Specifically it was used to determine the AVP responsiveness of clonal cell lines and the desensitisation of this response following a pre-treatment. This assay is a cell based assay and as such requires a number of wash steps, particularly in determining the desensitisation of the response (see Section 2.9). HEK293 cells exhibit poor adherence when subjected to a number of washes within a short time frame. A number of factors within the assay were assessed in order to optimise HEK293 cell adherence including:

1) culture-ware coatings,
2) protein source added to the media,
3) wash media, and
4) technique of media replacement

The effects of changes made to these parameters were visually assessed by evaluating the percentage confluence and cell morphology before and after the washes. The changes that enhanced cell adherence were then incorporated into the final protocol which can be seen in Section 2.9.

3.6.2 RESULTS

3.6.2.1 Assessment of different cell culture-ware coatings

Due to the number of washes in the IP assays, cell culture-ware coated to promote cell adhesion must be used with HEK293 cells. Several plate coatings used to promote cell adhesion were assessed, these included poly-D-lysine (PDL), Amine, Carboxyl and Primaria (Becton Dickson).

The different plate coating types were assessed in a number of trial experiments using both untransfected and transiently transfected HEK293 cells. The transfected cells were slightly more fragile than the untransfected cells and as such were observed to lift off the cultureware

more readily then the (untransfected) HEK293 cells. This was taken into account when assessing the results from these experiments. In these trial experiments some changes in morphology were seen after wash steps. This was usually an indicator of reduced adherence. When conducting these trial experiments the HEK293 cells never exceeded 100% confluence. This ensured that the results were not negatively impacted by cell growth phase. Table 3.4 summarises the results from transiently transfected cells assessed on the different plate coating types.

Plate type	Final Morphology	Final Confluence	Percentage Stimulation
PDL	Changed	~20%	159%
Amine	Changed	~20%	262%
Carboxyl	Normal	~70%	122%
Primaria	Normal	~70%	197%

Table 3.4 Summary of the results from different plates coatings with transiently transfected cells. The final morphologies and confluences were determined after all of the washes described in Section 2.9.3.1.2. The percentage stimulation was determined after the washes described in Section 2.9.3.1.1.

When HEK293 cells were grown on PDL and Amine coated plates they exhibited a change in morphology from normal, after the pre-label wash and the addition of the labelling medium. This change in morphology was generally associated with a lower level of adherence, which resulted in variable numbers of cells remaining in the wells after all the washes. This variability produced unreliable results from these plates. The change in morphology started at the edge of the wells where gaps would form in the cell monolayer. The cells then started to group together in mounds. While this was occurring the gaps became more pronounced and the cells at the edges of the gaps in particular became rounded rather than flattened. Sometimes during media changes these mounds lifted from the plate surface and formed balls of cells which collected in the media at the centre of the wells. On the Amine coated plates the extent of the morphological changes were exacerbated by higher concentrations of FBS, and as a consequence cells were plated onto these plates in 5% FBS, rather than 10%. Examples of the changed morphology can be seen in Figure 3.14. The reproducibility of the values obtained for AVP stimulated IP production in cells plated on Amine coated plates was only

acceptable when the protocol did not involve the extra washes required when desensitisation was being measured.

HEK293 cells that were plated onto Carboxyl coated plates had a different morphology from normal initially. This changed to a normal morphology usually the day after plating. HEK293 cell adherence on this type of plate coating was of an acceptable level. However, the cell growth rate on these plates was slower than that seen on the other types of plate coating. The AVP responsiveness of the cells plated on this sort of coated plate was unacceptably low.

Untransfeceted and transfected HEK293 cells exhibited high levels of adherence along with an unchanged morphology on Primaria coated plates. This level of adherence provided consistent and reliable numbers of cells to work with. This reliability allowed for significant differences to be seen between treatments. The responsiveness of the cells to AVP on this type of plate was of a workable level and was reproducible. The reproducibility of results on these coated plates made them a good choice for the IP assay experiments.

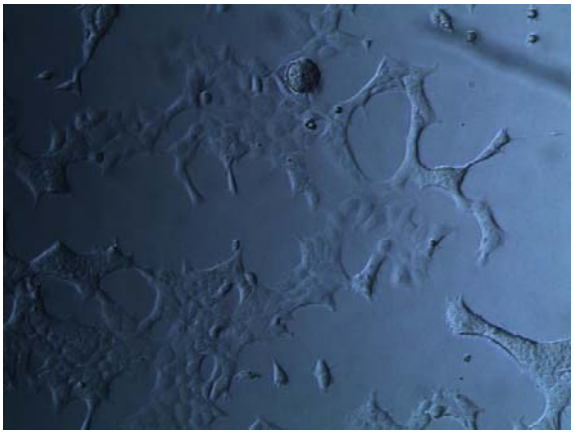

Figure 3.14 Change in morphology seen with HEK293 cells. This image was taken of cells grown on a PDL coated plate. Gaps have formed with rounded edges. The cells can be seen to be mounding up around the edges of the gaps. This is most pronounced on the right hand side of the image.

3.6.2.2 Optimisation of selected IP assay solutions

Preliminary experiments using 1x PBS for the wash medium prior to labelling, lowered the levels of cell adherence. Therefore, it was decided to change the wash medium to inositol free DMEM. This allowed the cells to be washed clean of inositol prior to labelling while

maintaining a gentler wash medium. A greater level of cell adherence was seen after this change in wash medium. Therefore this change was permanently incorporated into the protocol.

The cells were plated for the IP assay in 10% FBS. However, to improve reproducibility in the functional assays any serum should be removed from the medium prior to the assay. It was therefore decided to change the protein source in the labelling medium from FBS to bovine serum albumin (BSA). As BSA was already the standard protein source for the AVP stimulation and pre-treatment steps, it was therefore a suitable choice to test. This change to the labelling medium reduced the morphology changes seen, as well as increasing the level of cell adherence.

3.6.2.3 Optimisation of media removal and addition to maintain cell adherence and normal morphology

To ensure that the wells spent as little time as possible without medium during the changes, no more than twelve wells were handled at one time. If the experiment required more than twelve wells, more than one plate was plated. These plates were staggered 3 h apart. This was done because the lifting of the cell monolayer occurred to a greater extent the longer the cells were without medium. To further address this issue the medium was changed in six wells at a time. For example the media was removed off the first six wells and the wash added. The media was then removed from the next six wells and the wash added. The wash and the new solution were removed and added in the same stepwise manner. The media and solutions were all removed from the wells via aspiration. The new media and solutions were added to the well using a P1000 Gilson pipette with the tip against the side of the well. These solutions were always added in one smooth motion, as a “stop-start” motion lead to a decrease in cell adherence.

CHAPTER 4:

SCREENING AND ASSESSMENT OF V1BR-STABLY TRANSFECTED HEK293 CELL LINES

4.1 INTRODUCTION

The V1bR plays an important role in the regulation of the HPA axis, which controls an individual's response to stress (see Chapter 1 for details). Understanding how the V1bR is regulated is an important step to understanding adaptation to stress (Aguilera, 1994). Previous work using ovine anterior pituitary cells has shown that the V1bR desensitises in response to repeated AVP stimulation, and that this desensitisation occurs at concentrations and durations of AVP exposure which fall within endogenous ranges (Hassan et al., 2003). A pre-treatment with 5 nM AVP for 5 min significantly reduced the ACTH response to AVP to 78.5% of the control response. This indicated that V1bR desensitisation is likely to be a mechanism of receptor regulation *in vivo*. Since receptor desensitisation appears to be involved in receptor regulation *in vivo*, a research focus in the Mason laboratory involves deducing the molecular mechanisms of desensitisation of the V1bR.

In the classical model of GPCR signalling, desensitisation is produced from a combination of mechanisms including uncoupling of the receptor from its signalling pathway as a result phosphorylation, receptor internalisation, and down regulation of the receptor (Ferguson, 2001; Lohse, 1993) (see Chapter 1 for details). These mechanisms occur over different time scales. Phosphorylation of the receptor results in rapid (within seconds) and reversible desensitisation (Ferguson, 2001). The magnitude of desensitisation mediated by phosphorylation with PLCβ coupled receptors is variable (Wojcikiewicz et al., 1993). Maximum desensitisation is achieved through a combination of all of the mechanisms. Previous work in the Mason laboratory has ruled out PKC and CK1α as potential phosphorylators in V1bR desensitisation (Hassan and Mason, 2005). This research did indicate that PP2B was involved in resensitisation of the receptor, which has previously been shown to be involved with the resensitisation of the β_2AR when it is desensitised by GRK2 (Shih et al., 1999). The GRK family are common GPCR phosphorylators (Pitcher et al.,

1998), and therefore, GRKs are currently thought to be the most likely candidates for V1bR phosphorylation.

RNAi is the method of choice for loss of function studies. This is because RNAi, the siRNA form in particular, is excellent at degrading target mRNA and therefore 'knocking down' the mRNA and protein expression of specific genes (Editorial, Nature Cell Biology, 2003). The use of siRNA techniques means primary cell cultures are an inappropriate choice as they are difficult to transfect and their heterogenous nature makes interpretation of biochemical assays very difficult (Freshney, 2000; Grimm, 2004; van Beijnum et al., 2008). Therefore, the decision to use siRNA necessitated a change from use of primary cell cultures to a model cell system using an immortalised cell line, which provided an easily transfectable homogenous cell population. The development of a model cell system stably-transfected with the V1bR was therefore the goal of this project. HEK293 cells are a good option for the development of a model system as, in addition to being easy to transfect, the sequences of human GRKs are known, and specific siRNAs have been developed to target them (Ren et al., 2005). These siRNAs have been used previously in HEK293 cells to knockdown and examine the role of specific GRKs in phosphorylation of the V2R.

The preparation for and the development of the model cell system was described in Chapter 3. This Chapter focuses on the partial characterisation of the generated clonal cell lines. Once characterisation is completed, these cell lines will provide a homogenous pool of cells suitable for detailed investigation of the molecular mechanisms of V1bR signalling and its regulation.

4.2 INITIAL SCREENING OF CLONAL CELL LINES TO IDENTIFY THOSE RESPONDING TO AVP

4.2.1 INTRODUCTION

Due to the random nature of DNA integration (Mortensen and Kingston, 2009), different clonal cell lines can have different levels of gene expression. It is also possible that even though the resistance gene is expressed, the GOI is not. This meant that after selection, all ten of the established clonal cell lines required individual assessment to determine whether they expressed functional V1bRs. This assessment of the cell lines utilised the IP assay.

The IP assay can be used to assess signalling and desensitisation for any PLCβ coupled GPCR in an intact cell system. The desensitisation of the IP response can also be assessed following

an agonist pre-treatment. This assay has been used previously with HEK293 cells to assess signalling with a number of GPCRs including; the human endothelin receptors A and B (Freedman et al., 1997), the type 1A angiotensin II receptor (Oppermann et al., 1996), and metabotropic glutamate 1A receptor (Dale et al., 2000). The IP assay has also been previously used to examine V1bR signalling and desensitisation (Gatehouse, 2008; Rabadan-Diehl et al., 2007; Thibonnier et al., 1997; Young et al., 2007).

The measurement of IP production in response to AVP stimulation allows for a number of factors involved with V1bR signalling to be examined including:

1) the responsiveness of the cell lines to AVP stimulation,
2) the magnitude of response desensitisation following a pre-treatment with AVP, and
3) any changes in stimulated IP production over a number of passages.

Characterisation of these signalling parameters is necessary, in order to provide a baseline for subsequent work with this model cell system.

The IP production of the clonal cell lines in response to AVP stimulation was used as an indicator of rV1bR expression. Initially all of the lines were screened for increased IP production in response to AVP (100 nM, 15 min) using the IP assay (see Section 2.9). This screen allowed for identification of individual clonal cell lines that respond to AVP. The AVP responsiveness and the desensitisation of this response following an AVP (5 nM, 5 min) pre-treatment of the identified cell lines were then examined in greater detail (see Section 4.3).

4.2.2 RESULTS

Ten clonal cell lines were selected using G418 and established as individual lines. These were then screened for expression of either EGFP or rV1bR. The one clonal cell line that was transfected with pEGFP-N1 was assessed for EGFP expression using fluorescence confocal microscopy. The nine clonal cell lines that were transfected with pN1-V1bR were screened for functional expression of the rV1bR by assessing intracellular IP production in response to AVP (100 nM) stimulation for 15 min (see section 2.9.3.1.1 for details). Briefly, this involved washing the cells that had been incubated for 20 h with *myo*-[^{3}H]inositol once with the "basal solution" and then incubating the cells with either "basal solution" (0 nM AVP) or "stimulation solution" (100 nM AVP) for 15 min. This allowed AVP stimulated IP production to be compared to the basal level of IP production.

The day after transfection, the cells transfected with pEGFP-N1 were assessed using fluorescent confocal microscopy. A large number of the cells were shown to be expressing EGFP (data not shown). See Section 3.4 for an example of images of fluorescing cells. However, when the stable clonal cell line established from cells transfected with pEGFP-N1 was assessed, it did not express EGFP. No assessment of expression was made between these two time points.

Nine clonal cell lines were generated from the cells transfected with pN1-V1bR. These cell lines were all initially screened once with the IP assay with all treatments in triplicate, in order to assess their responsiveness to AVP. Four of the nine clonal cell lines assessed responded to AVP stimulation with an increase in IP production of ~50% or more (Table 4.1). This indicated that the rV1bR was probably expressed in these four clonal cell lines. Of the four lines, 2A5-210210 and 1C5-210210 (referred to from here on as 2A5 and 1C5, respectively), appear to have a greater responsiveness to AVP, than 1C1-210210 and 2C2-210210 (referred to from here on as 1C1 and 2C2, respectively). Of the five remaining lines, 2A1-210210 cells were too fragile to survive a single freeze-thaw cycle. Therefore, AVP responsiveness could not be assessed in this line. The other four lines, 1A5-210210, 2B2-210210, 2B4-210210, and 1B1-210210, had insufficient levels of AVP responsiveness for them to be considered for further characterisation.

Clonal cell lines	IP response (% response of 0 nM AVP)
2A5-210210	253%
1C1-210210	168%
1A5-210210	100%
2B2-210210	122%
2C2-210210	148%
2A1-210210	-
2B4-210210	108%
1C5-210210	286%
1B1-210210	110%

Table 4.1 Screening of the established clonal cell lines. The AVP stimulated IP response is expressed as a percentage of the basal response.

The four cell lines that showed an increase in IP production in response to AVP stimulation were further characterised. This included assessments of both the response to AVP and the desensitisation of this response following a 5 min AVP (5 nM) pre-treatment.

4.3 PARTIAL CHARACTERISATION OF V1bR-STABLY TRANSFECTED HEK293 CELL LINES

4.3.1 INTRODUCTION

The four clonal cell lines, shown by their responsiveness to AVP to express the V1bR, were partially characterised in greater detail. Within the time frame of this thesis, the main focus for characterisation of these lines was on determining the presence of a functional receptor. In this case, this meant determining if the external signal (AVP stimulation) was successfully received and transduced across the plasma membrane. Successful reception and transduction were measured as an increase in IP production. The cell lines were also assessed to see if they were capable of undergoing desensitisation following an AVP pre-treatment. This was of particular importance, since this is a normal aspect of V1bR function and is currently under investigation in this laboratory. Both of these parameters were examined using the IP assay (see Section 2.9).

A different wash and AVP treatment protocol was used for these experiments than that which was used for screening purposes. This change allowed for both the IP response to a 15 min 100 nM AVP treatment to be determined along with the change in the IP response following a pre-treatment for 5 min with 5 nM AVP (see Section 2.9.3.1.2 for full details). Briefly, after the 20 h *myo*-[^{3}H]inositol incubation the cells were washed once with "control solution", and then incubated with either "control solution" (0 nM AVP) or "pre-treatment solution" (5 nM AVP) for 5 min. After this pre-treatment the cells were washed twice with "basal solution" and then incubated with either "basal solution" (0 nM AVP) or "stimulation solution" (100 nM AVP) for 15 min.

4.3.2 RESULTS

4.3.2.1 IP response of the clonal cell lines to AVP stimulation

All four of the clonal cell lines, selected from the screening process exhibited large (>300% at the first passage) increases in the levels of IP production in response to stimulation with AVP (Table 4.2). This indicates that all of these cell lines express the rV1bR. IP levels were

significantly increased by AVP stimulation in both first and tenth passage cells (Figure 4.1). The stimulated IP responses for all of the four cell lines from passage one and passage ten (as indicated in Table 4.2) were compared. Clonal cell line 1C5 exhibited a larger IP response to AVP stimulation at both passage one (P<0.01; One way ANOVA with Bonferroni's test) and passage ten (P<0.01; One way ANOVA with Bonferroni's test) than the other three cell lines. The IP responses were ~500% and ~320% of the basal response at the first and tenth passage respectively. AVP stimulated IP production decreased significantly between the first and tenth passages (Table 4.3). This decrease was approximately one third for all of the clonal cell lines between passages one and ten.

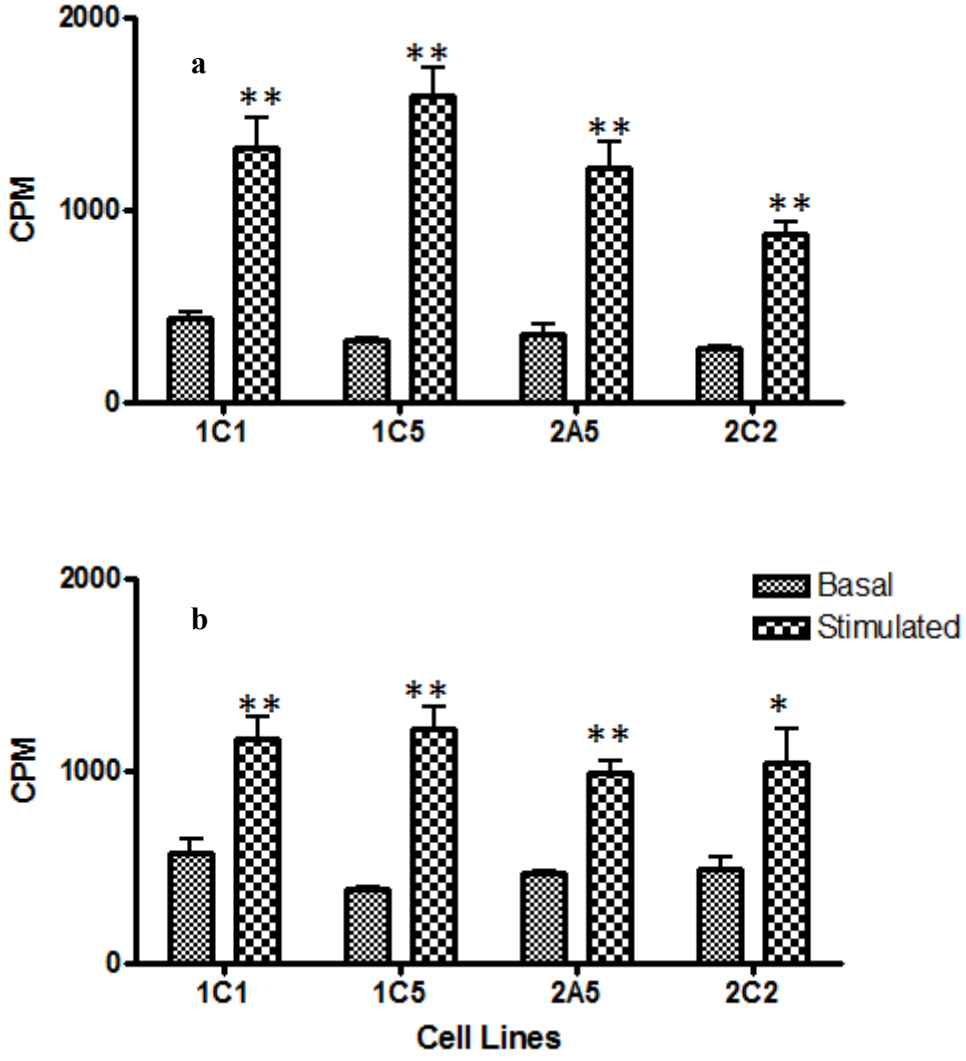

Figure 4.1 IP responses of cells treated with 0 nM ("basal") or 100nM ("stimulated") AVP. All of the cells lines exhibited a significant increase in IP production in response to AVP stimulation at both the first passage (a) and tenth passage (b). This increase in IP production indicates that the cell lines are expressing the rV1bR. The data are expressed as mean ± SEM (n=3). Asterisks indicate a statistically significant difference between the basal and stimulated responses for each cell line (t-test; *P<0.05; **P<0.01).

		1C1	1C5	2A5	2C2
First passage	Basal CPM	472.2 ± 29.9	320.9 ± 8.9	353.1 ± 50.2	277.2 ± 16.9
	Stimulated CPM	1327.0 ± 147.4	1597.0 ± 137.1	1215.7 ± 139.5	876.0 ± 63.4
	Stimulated IP response	□□(Ausubel et	499.9 ± 51.9%	347.1 ± 12.7%	315.6 ± 4.3%
	P value	0.0089	0.0062	0.0054	0.0030
Tenth passage	Basal CPM	575.0 ± 73.5	308.9 ± 17.1	465.8 ± 15.0	486.1 ± 63.0
	Stimulated CPM	1163.3 ± 114.6	1222.0 ± 111.9	991.4 ± 58.8	1041.6 ± 172.2
	Stimulated IP response	203.9 ± 9.3%	320.0 ± 20.1%	212.6 ± 6.8%	212.3 ± 7.6%
	P value	0.0039	0.0068	0.0037	0.0184

Table 4.2 IP responses of cells treated with 0 nM ("basal") and 100 nM ("stimulated") AVP. The results from both passage one and ten cells are presented for all four cell lines. All of the clonal cell lines exhibit a significant increase in IP production in response to AVP stimulation. This indicates that all of the cell lines are expressing the rV1bR. The "basal" and "stimulated" IP response data are expressed as CPM. The stimulated IP response is the stimulated CPM as a percentage of the basal CPM. Data are mean ± SEM (n=3). One way paired *t*-tests were performed to determine the significance of the difference between the basal and stimulated CPM. A *P* value of <0.05 was considered significant.

	1C1	1C5	2A5	2C2
First passage stimulated IP response	302.0 ± 17.8%	499.9 ± 51.9%	347.1 ± 12.7%	315.6 ± 4.3%
Tenth passage stimulated IP response	203.9 ± 9.3%	320.0 ± 20.1%	212.6 ± 6.8%	212.3 ± 7.6%
Change in stimulated response	67.8 ± 4.9%	64.8 ± 5.0%	61.5 ± 4.1%	67.3 ± 2.2%
P value	0.0082	0.0318	0.0007	0.0003

Table 4.3 Comparison of the AVP stimulated IP production between passage one and ten for the four cell lines. There is a significant decrease for all cell lines in AVP stimulated IP production between passage one and ten cells. Data are expressed as percentage changes in IP responses, mean ± SEM (n=3). Two way un-paired *t*-tests were performed to determine the significance of the difference between the stimulated response with first and tenth passage cell. A *P* value of <0.05 was considered significant.

4.3.2.2 Desensitisation of the cells to AVP stimulation

All of the clonal cells lines exhibited a significant decrease in IP production in response to AVP stimulation following an AVP pre-treatment (Table 4.4). This decrease in IP production shows desensitisation of the cells response to AVP stimulation following an AVP pre-treatment, which indicates desensitisation of the V1bR. Only lines 1C5 and 2A5 exhibited a

significant decrease in AVP responsiveness with both first and tenth passage cells (Figure 4.2). The pre-treated response of clonal cell line 1C5 was 60.1 ± 4.7% and 69.1 ± 3.8% that of the control response for first and tenth passage cells respectively. The pre-treated response of 2A5 was 65.2 ± 4.6% and 77.1 ± 4.9% of the control response for first and tenth passage cells respectively. With clonal cell lines 1C1 and 2C2, the decrease in AVP responsiveness was only significance with tenth passage cells. The pre-treated responses at the tenth passage were 79.6 ± 4.5% and 84.9 ± 2.9% of the controls, respectively.

No significant differences in the magnitude of desensitisation between first and tenth passage cell were seen with any of the clonal cell lines (Table 4.5).

		1C1	1C5	2A5	2C2
First passage	Control IP response	302.0 ± 17.8%	499.9 ± 51.9%	347.1 ± 12.7%	315.6 ± 4.3%
	Pre-treated IP response	242.7 ± 45.9%	296.1 ± 17.3%	225.0 ± 9.0%	273.6 ± 25.1%
	Change in IP response	79.1 ± 10.8%	60.1 ± 4.7%	65.2% ± 4.6%	86.7 ± 7.9%
	P value	0.0866	0.0221	0.0134	0.1193
Tenth passage	Control IP response	203.9 ± 9.3%	319.9 ± 20.2%	212.6 ± 6.8%	212.3 ± 7.8%
	Pre-treated IP response	161.4 ± 3.4%	220.8 ± 15.3%	164.2 ± 13.6%	180.7 ± 12.2%
	Change in IP response	79.6 ± 4.5%	69.1 ± 3.8%	77.1 ± 4.9%	84.9 ± 2.9%
	P value	0.0321	0.0107	0.0204	0.0150

Table 4.4 IP responses of cells stimulated with AVP, with or without an AVP pre-treatment. Only clonal cells lines 1C5 and 2A5 showed a significant reduction in IP response to AVP stimulation following an AVP pre-treatment with both first and tenth passage cells. Lines 1C1 and 2C2 only exhibited a significant reduction with passage ten cells. The data are expressed as percentage changes in the IP response compared to a control, mean ± SEM (n=3). One way paired *t*-tests were performed to determine the significance of the difference between the control and pre-treated IP response, a *P* value of <0.05 was considered significant.

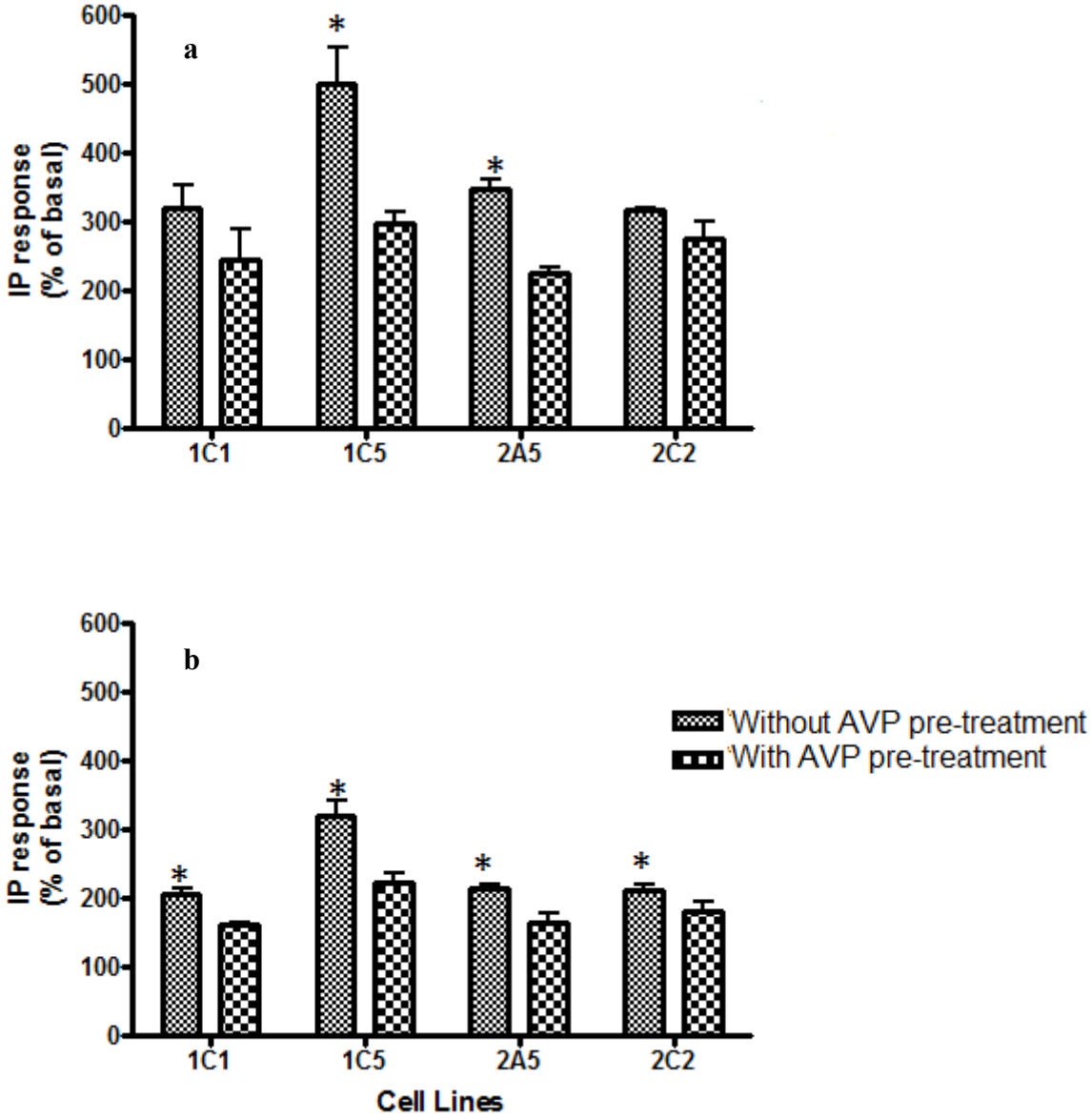

Figure 4.2 IP responses of cells stimulated with AVP, with or without an AVP pre-treatment. a). At the first passage, only cell lines 1C5 and 2A5 show a significant reduction in the IP response to AVP (100 nM, 15 min) stimulation following an AVP (5 nM, 5 min) pre-treatment. b). At the tenth passage, all of the clonal cell lines showed a significant reduction in IP response to AVP stimulation following an AVP pre-treatment. The results are expressed as the AVP stimulated IP response as a percentage of the basal response. Data are expressed as mean ± SEM (n=3). Asterisks indicate a statistically significant difference between IP production in cells that were pre-treated with AVP and those that were not (*t*-test; **P*<0.05).

	1C1	1C5	2A5	2C2
First passage desensitisation	79.1 ± 10.8%	60.1 ± 4.7%	65.2% ± 4.6%	86.7 ± 7.9%
Tenth passage desensitisation	79.6 ± 4.5%	69.1 ± 3.8%	77.1 ± 4.9%	84.9 ± 2.9%
P value	0.9726	0.2038	0.1496	0.8420

Table 4.5 Comparison of the magnitude of desensitisation between first and tenth passage cells. None of the clonal cell lines show a significant difference between the magnitude of desensitisation seen with the first and tenth passage cells. Data are expressed as the magnitude of desensitisation. The desensitisation was calculated as the AVP pre-treated response as a percentage of the control pre-treated response, mean ± SEM (n=3). Two way un-paired *t*-tests were performed to determine the significance of the difference between the magnitude of desensitisation seen at the first and tenth passages. *P* values of <0.05 were considered significant.

4.3.2.3 General growth characteristics of the clonal cell lines

Some general differences between the four clonal cell lines were observed while culturing the cells for the IP assays. Line 2A5 grew at a consistently faster rate than the other three. When the line was passaged the new flask was seeded at 6,000 cells per cm^2, in order to reach 50-80% confluence after 4 days of culturing. This seeding density was comparable with the seeding density of the parental HEK293 cells. These were seeded in flasks at 5,200 cells per cm^2 in order to reach 50-80% confluence after 4 days. The lines 1C5 and 1C1 grew at a slightly slower rate than 2A5, and were seeded in flasks at 9,200 cells per cm^2 in order to reach 50-80% confluence after 4 days. The growth rate was approximately the same between the two lines. Line 2C2 grew at the slowest rate of all four lines and required the flasks to be seeded at 12,000 cells per cm^2 in order to reach 50-80% confluence after 4 days. This is double the plating density required for line 2A5. In general, the clonal cell lines seemed to have a lower level of adherence than the parental HEK293 cells, as they tended to lift in greater numbers during the washes in the IP assay.

The other major observable difference with the cell lines was that 1C5 cells were slightly larger (Figure 4.3) than the other three lines, which appear to be of a similar size to the parental HEK293 cells (see Figure 2.1). This difference in size was most notable when the cells were being counted, as the individual 1C5 cells would appear much larger on the haemocytometer grid than the cells from the other clonal cell lines. As a consequence the cell counts for 1C5 were consistently lower than expected from the estimation of confluence.

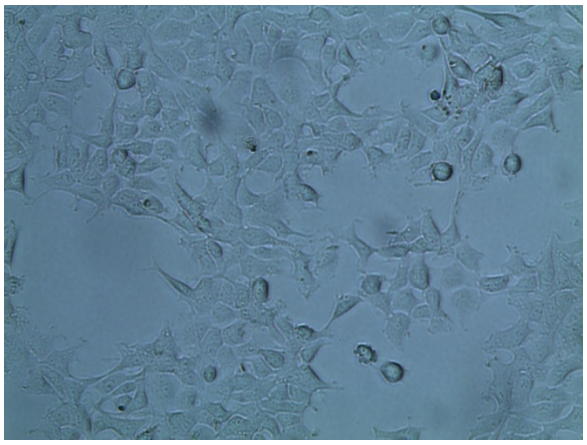

Figure 4.3 Image showing clonal cell line 1C5 morphology. Cells of the clonal cell line 1C5 appear slightly larger than those from the other three clonal cell lines. These other three clonal cell lines were similar in size to the parental HEK293 cell line, which can be seen in Figure 2.1.

CHAPTER 5:

DISCUSSION

5.1 SUMMARY

The main goal of this project was to develop a stably-transfected model cell system that could be used to investigate the molecular mechanisms of V1bR desensitisation. In addition to generation of V1bR-stably transfected cell lines, this research involved both preparatory work and a partial characterisation of four pN1-V1bR expressing lines.

Preparation and planning ensured that a model cell system appropriate for examining the molecular mechanisms of V1bR desensitisation was developed. Several parameters were examined and optimised to ensure the successful completion of this project, in particular:

1) the choice of parental cell line,
2) cell growth phases and the effect of this upon experimental results,
3) plasmid design and purification,
4) the choice and optimisation of a selection marker, and
5) the choice and optimisation of a transfection reagent.

Many facets of these parameters are inter-related and therefore had to be optimised to work cohesively within the framework of this project to ensure its successful outcome (see Chapter 3 for full details). Briefly, HEK293 cells were chosen as a parental cell line, as this cell line can be transfected easily and is appropriate for future work with siRNA. Determining the cell growth rates and phases enabled more precise experimental planning for the later experiments in this research. This greater precision in experimental planning helped to ensure the successful completion of experiments and maintained the health of the cultured cells. Plasmid sub-cloning was utilised so that the GOI and the selection marker were included on the same plasmid. Sub-cloning also simplified the transfection protocol as a co-transfection was not required. This also provided greater confidence, during selection, that if the cells were resistant to G418 that they would also express the GOI, V1bR or EGFP. Purification of the plasmids was an important step as successful transfection of mammalian cells requires highly pure DNA. As can be seen from the results in Chapter 4, the plasmid DNA produced was of a

sufficiently high quality to successfully transfect mammalian cell lines. The choice of mammalian antibiotic used for selection was made as a consequence of the selection marker available, *neo*. This gene provides not only eukaryotic cells with resistance to G418, but it also provides prokaryotic cells with resistance to neomycin and kanamycin (Southern and Berg, 1982). This means that *neo* can be used for selection in plasmid production in *E. coli* as well as selection of stably-expressing mammalian cell lines. Optimisation of G418 selection conditions determined 500 μg/ml as an appropriate selection concentration, and 250 μg/ml as an appropriate maintenance concentration. This was in the mid range of the concentrations tested. G418 was easy to use and successfully killed all untransfected cells, as was determined by the 100% death rate of the untransfected negative control for the stable transfection (see Section 3.5). F6 was chosen as the transfection reagent for this project, as it has a straightforward transfection protocol, works on cells with a lower percentage confluence than many other similar reagents, and produces only low levels of cytotoxicity (Jacobsen et al., 2004). The F6:DNA (μl:μg) ratio, the complex formation time and the time required for expression of the gene were optimised, as per the manufacture's instructions. The optimised F6 transfection protocol was successfully used in the generation of the clonal cell lines (see Sections 3.4 and 3.5).

Once these parameters were optimised, a model cell system stably-expressing the pN1-V1bR could be developed. A total of ten clonal cell lines (transfected with either pEGFP-N1 or pN1-V1bR) were selected using G418 and established as individual clonal cell lines. Nine of these lines were established from cells transfected with pN1-V1bR and one from cells transfected with pEGFP-N1. Cells transfected with pEGFP-N1 were used as a positive control to ensure that transfection had been successful, prior to G418 selection. Details of the key points in the development of these stably transfected cell lines can be seen in Section 3.5.

Due to the time constraints imposed by MSc. degree requirements only AVP responsiveness was used to characterise the established clonal cell lines. This characterisation was chosen for evaluation because it is the key indicator of functional expression of the receptor. The clonal cell line established from cells transfected with pEGFP-N1 was assessed using fluorescence confocal microscopy, so that EGFP expression could be determined.

5.2 PARTIAL CHARACTERISATION OF THE GENERATED CLONAL CELL LINES

The day after transfection, a day before G418 selection began, the cells transfected with pEGFP-N1 were assessed using fluorescent confocal microscopy. A high number of these cells exhibited EGFP expression, which indicated successful transfection of the plasmid (data not shown). However, the clonal cell line established from these through G418 selection did not express EGFP. This is most likely because the generation of stable cell lines (unlike transient expression) requires integration of plasmid DNA and this happens in a random fashion (Mortensen and Kingston, 2009). Random integration may have resulted in some genes in the plasmid being expressed while others are not. Possible reasons for this include, position effects, where integration into hetero- versus eu- chromatin will effect the expression level (Wurm, 2004). The cell linearises the DNA before it is integrated, and the point at which the plasmid is cut can affect expression of a particular gene, as the plasmid may be cut in the middle of a gene. Transgenes can be silenced after integration, which may mean initially expression is exhibited, but this is lost over time. As only one clonal line was established for EGFP expression, most likely random chance resulted in that particular clonal cell line not stably expressing the GOI, even thought most cells were seen to be expressing EGFP prior to G418 selection.

To address this issue, homologous recombination can be used to introduce DNA at specific sites (Mortensen and Kingston, 2009). These systems are generally used in the development of transgenic animals, but the same principles can be applied to cell lines. One such system that can be utilised is a *lox* recombination vector which is designed to directly select Cre-mediated site-specific DNA integration at a *lox* target previously incorporated into the genome (Fukushige and Sauer, 1992). Cre is a recombinase protein that mediates site specific recombination between *loxP* sites. Using a system such as this, the point at which the DNA is inserted into the host genome is reproducible. All clonal cell lines generated will be subjected to the same position effects and will exhibit the same level of expression. This approach was not used for this project. As a consequence, integration of the plasmid DNA into the genome was unique in each line. This means that some of the lines may not express the GOI, even if they have exhibited expression of the selection marker. This is because selection is based on expression of *neo* only.

Nine clonal cells lines transfected with pN1-V1bR were selected using G418 and established as individual cell lines. These lines were designated as 1A5-210210, 1B1-210210, 1C1-210210, 1C5-210210, 2A1-210210, 2A5-210210, 2B2-210210, 2B4-210210, and 2C2-210210. Clonal cell line 2A1-210210 was too fragile to withstand a single freeze-thaw cycle. Therefore, only the eight remaining lines were screened for changes in IP production in response to AVP stimulation, which was used as an indicator of rV1bR expression. From these screening experiments four of the eight clonal cell lines, 1C1, 1C5, 2A5, and 2C2, appeared to respond to AVP stimulation. The magnitude of AVP responsiveness and the desensitisation of this response were then characterised in these four clonal cell lines in greater detail.

All four of these chosen clonal cell lines showed a significant increase in the magnitude of response to AVP stimulation (100 nM, 15 min) with both first and tenth passage cells (see Figure 4.1). This response was at least double the basal response with both first and tenth passage cells. With cell line 1C5 there was a 5-fold increase in production with first passage cells. The increased IP production in response to AVP stimulation in 1C5 was significantly larger than the other lines. The AVP response dropped somewhat between the first and tenth passages for all four lines. The decrease was approximately equivalent between the cells lines with the responses of the passage ten cells being about two thirds of the responses of the passage one cells. Due to the range of changes that can occur in cell lines over time, no future experiments are likely to use cells beyond passage ten. Therefore, this data characterises the total decrease in the AVP responsiveness that could be expected over the course of future experiments.

The decrease seen in the responsiveness of the cells is most likely a consequence of the way in which the DNA has integrated into the genome as a result of the transfection method used (Mortensen and Kingston, 2009). Carrier molecule methods of transfection can introduce multiple copies of the plasmid DNA at the same site. These arrays of similar sequences can lead to intra-chromosomal rearrangement, which can lead to a decrease the number of copies of the plasmid and therefore of the gene over time. This drop in copy number will lead to a drop in the level of expression. Other methods of transfection incorporate the plasmid DNA in different ways. For example electroporation usually introduces one copy of the DNA at a single site. However, there are other disadvantages associated with the other methods of transfection which make them a less desirable choice than carrier molecules (see Section 1.3.1). As carrier molecules were chosen as the transfection method a significant drop in the

level of gene expression over time is not unexpected. Importantly the results indicate that the V1bR was still measurable and reproducibly expressed, as the response to AVP stimulation is still significant. Therefore, regardless of the drop in the magnitude of the response, the stable cell lines can still be used to examine the molecular mechanisms of V1bR signalling up to passage ten.

Following a 5 min pre-treatment with 5 nM AVP, all four of the chosen clonal cell lines exhibited a significant decrease in IP production in response to a 15 min stimulation with 100 nM AVP. From the first passage experiments, lines 1C5 and 2A5 showed a significant drop in IP production in response to AVP stimulation following an AVP pre-treatment. This IP response was between 60-65% of the control response. The other two lines, 1C1 and 2C2, did not show significant desensitisation at the first passage. The tenth passage experiments showed significant levels of desensitisation of IP production in response to AVP stimulation following a pre-treatment in all four of the cell lines. The magnitude of the responses ranged between 69-85% of the control responses, with only cell line 1C5 showing responses that were <70% of the control responses. The magnitude of desensitisation was not significantly different between the first and tenth passage cells for any of the lines. This was true even for 1C1, and 2C2. It is possible for lines 1C1 and 2C2 that a greater number of replicate experiments may be required to see a significant level of desensitisation at the first passage. This is because the magnitudes of desensitisation appeared similar at both passages, but there was a higher standard error for the results from the first passage.

It is desirable when choosing a cell line for investigating the molecular mechanisms of V1bR desensitisation that the line has similar signalling properties to the original tissue as this will increase confidence when exptrapolating details. Perifused ovine anterior pituitary cells previously examined in this laboratory exhibited an ACTH response to AVP stimulation 78.5 ± 1.5% of the control following an AVP (5 nM, 5min) pre-treatment (Hassan et al., 2003). A number of factors do need to be considered when comparing these results directly to those from the clonal cell lines. The receptors examined in the experiments were from different species, ovine V1bR in the perifusion studies (Hassan et al., 2003) and the rat V1bR (Lolait et al., 1995) used in this research. Different molecules were used to quantify signalling, ACTH secretion (Hassan et al., 2003) and IP production (this research). As there are a number of signalling steps between IP production and ACTH secretion any one of these may have affected the measurements. Different protocols were also used to obtain the data, and may have affected the results obtained. However, given the difference, all of the clonal

cells lines produced results that were remarkably similar to those seen with the perifused ovine anterior pituitaries. The lines all showed partial desensitisation within 5-15 min following a low (5 nM) AVP pre-treatment. Since the clonal cell lines exhibit slightly different properties, the line most suited to examining a particular question will be able to be utilised. This will provide greater flexibility in experimental design.

A number of other *in vitro* studies involving rat, mouse, ovine, and equine systems and *in vivo* studies with rats have also shown desensitisation of the V1bR in response to prolonged AVP stimulation (reviewed in Mason et al., 2002). These studies have in general measured either ACTH as the output of the signalling pathway, or AVP binding properties. As well as measuring different outputs, these experiments were generally conducted over longer time scales, for example AVP pre-treatments of hours not minutes. This means that the desensitisation seen in many of these experiments may involve internalisation and down-regulation rather than, or in addition to, receptor G-protein uncoupling, which generally occurs within seconds (Ferguson, 2001). So the studies focusing on receptor desensitisation that are most relevant for comparison with the results obtained using the V1bR-stably transfected HEK293 cells are those in which perifusion ovine anterior pituitary cells were used (Hassan et al., 2003; Hassan and Mason, 2005) to look at regulation via rapid uncoupling.

The results presented here describing AVP responsiveness and desensitisation, indicate that all the clonal cell lines should be further characterised. However, 1C5 and 2A5 appear to have the greatest potential as model cell systems for examining the molecular mechanisms of desensitisation for a number of reasons. Clonal cell line 1C5 has a significantly greater stimulated IP response than the three other lines ($P<0.01$; One way ANOVA with Bonferroni's test). The actual numbers of receptors must be determined before assigning this difference to differences in receptor expression. This is because it is possible that the difference in stimulated IP response may be due to differences in any of a number of steps in the PLCβ mediated signalling pathway (Alberts et al., 2002). Therefore, absolute numbers of expressed receptors need to be determined through binding assays and Scatchard plot analysis. It would be interesting to investigate signalling mechanisms in cell lines with different expression levels of the V1bR as different signalling pathways have been shown previously to be activated in cells expressing the receptor at different levels (Thibonnier et al., 1997). From the results of the first passage experiments, both 1C5 and 2A5 showed a consistent level of AVP desensitisation to <70% of the control response. This consistent and

considerable magnitude of desensitisation will be important when trying to determine if the knock down of specific GRKs has any affect on the magnitude of desensitisation and hence determine which kinase is responsible for uncoupling. A considerable and consistent magnitude of desensitisation will be important as knocking down GRKs may only produce small changes in desensitisation. These changes are more likely to be noticeable if the standard desensitisation is consistent and of a considerable magnitude. The clonal cell line 2A5 exhibited the growth rate closest to that of the parental (untransfected) HEK293 cell line. This indicates that integration of the plasmid DNA into this cell line has been the least disruptive to normal cellular function.

5.3 V1bR-STABLY TRANSFECTED HEK293 CELL LINES AS A MODEL CELL SYSTEM

5.3.1 POTENTIAL USES

After full characterisation of the clonal cell lines, they will be valuable as model systems to dissect the V1bR signalling pathway and its regulation at the molecular level. In the Mason laboratory they will be used initially to examine the mechanisms involved with V1bR desensitisation.

The desensitisation process of GPCRs involves a combination of mechanisms including phosphorylation of the receptor which uncouples the receptor from its signalling pathway, internalisation of the receptor, and down regulation of the receptor (Ferguson, 2001; Lohse, 1993). These mechanisms occur over different temporal scales, phosphorylation of the receptor occurs rapidly (within seconds) and in a reversible manner. The results of pervious research have ruled out a number of possible phosphorylators including PKC and CK1α, and have demonstrated the involvement of PP2B in the resensitisation process (Hassan and Mason, 2005). PP2B had earlier been shown to be involved with the resensitisation of the β_2AR, when it was desensitised by GRK2 phosphorylation (Shih et al., 1999). GRKs are also known to be a common phosphorylator of GPCRs (Pitcher et al., 1998), and therefore, GRKs are currently thought to be the most likely candidates for phosphorylation of the V1bR. Only four of the seven GRKs (GRKs -2, -3, -5, and -6) have the appropriate distribution to make them possible candidates (Ferguson, 2001; Pitcher et al., 1998). The potential involvement of all four should be examined as there appears to be some level of redundancy (Pitcher et al., 1998). However, there is also the possibility that the different groups of GRKs (GRKs -2 and

-3, and GRKs -5, and -6) have different functional signalling consequences (reviewed in Reiter and Lefkowitz, 2006) as has been seen with other GPCRs, including the angiotensin II receptor (Kim et al., 2005), and the V2R (Ren et al., 2005). This potential for functional differences in specific GRK signalling should be examined so that a full picture of GRK involvement in the molecular mechanism of V1bR desensitisation can be obtained.

This model cell system could also be utilised to investigate of other aspects of V1bR signalling, including internalisation and down regulation, re-sensitisation and transport back to the plasma membrane. Internalisation and recycling of the receptor could be monitored by fluorescence microscopy (reviewed in Daly and McGrath, 2003). Fluorescent monitoring is particularly useful as the same cells can be assessed over a variety of time points. To do this a fluorescent V1bR agonist that will internalise with the receptor would be required for this to be considered a feasible option. Down-regulation could be determined through receptor binding assays, with cells assayed for receptor numbers before and after a prolonged exposure to AVP. As down-regulation of receptor content can take hours to occur (Ferguson, 2001), the length of AVP stimulation would have to be much longer than that used to assess signalling and desensitisation in this research (see Section 2.9).

The effects of AVP concentration on signalling and desensitisation could also be investigated. The concentration of AVP has already been shown to effect V1bR signalling in transiently transfected HEK293 cells (Orcel et al., 2009). There, higher concentrations of AVP resulted in signalling via cAMP as well as IP second messenger systems. Investigations into if and how utilisation of different pathways affects desensitisation, specifically phosphorylation, could be completed. Desensitisation of the receptor and the molecular mechanisms involved could be assessed using different AVP stimulation protocols and measuring the accumulation of IPs (as per this research) or cAMP as described by Orcel et al., (2009).

Therefore, the model cell system developed in this project has a wide variety of potential uses. This makes it a valuable tool in the investigation of V1bR signalling and desensitisation. It is hoped that these investigations will then be able to provide insights into normal V1bR signalling and desensitisation within pituitary corticotroph cells.

5.3.2 LIMITATIONS

Although there are a variety of potential of uses for this model cell system, there are limitations which must be carefully evaluated. Good experimental design and planning, which takes these limitations into account, is crucial in ensuring valid results are obtained. These

results will then be able to be used as a basis for developing a model of the signalling and desensitisation mechanisms within pituitary corticotroph cells

It has been shown that intracellular molecules involved in signalling processes can often be affected by cellular context, including expression of GRKs and β-arrestins (Ferguson, 2001). These differences can affect the signalling and desensitisation mechanisms utilised by the GPCR under investigation, as it has been shown that GPCR phosphorylation is a complex process and it is possible to phosphorylate one receptor with a number of different kinases (Tobin, 2008). While this provides the potential to tailor signalling of the same receptor in different cells within an organism to cater for differing physiological roles, it may mean that the mechanism seen in these clonal cell lines is different from that in seen pituitary corticotroph cells, as the cellular context is different. Therefore, although this model cell system will provide valuable information on the likely mechanism of desensitisation, any mechanistic details should be confirmed with work in pituitary corticotroph cells. Investigations using corticotroph cells are associated with a number of inherent difficulties including limited numbers of divisions, greater difficulty to transfect, and a heterogenous population of cells (Freshney, 2000; Grimm, 2004; van Beijnum et al., 2008). Deducing a large amount of molecular mechanistic detail in the easier to use immortalised cell lines, will mean that focussed experiments that take these difficulties into account can be planned for the primary cell cultures in order to confirm these details.

The AVP receptors have been shown to have some species specific differences in interactions with different synthetic agonists and antagonists (Chini and Manning, 2007; Guillon et al., 2004; Guillon et al., 2006; Pena et al., 2007; Serradeil-Le Gal et al., 2002). Most of the synthetic agonists and antagonists were developed using receptors from the rat. When these synthetic peptides were then tested on human AVP receptors they were shown to have different binding affinities and pharmacological properties. However, the binding affinities of AVP appear to be conserved. As there are indications of some species specific differences in the receptors, this should be a consideration when extrapolating the molecular mechanisms of receptor desensitisation from one species to another. This consideration will be particularly important if differences in signalling and desensitisation are examined using synthetic agonists or antagonists.

The clonal cell lines developed in this project have yet to be fully characterised. This characterisation will provide more detailed information of receptor expression within the

model cell system. Comparison of the expression profiles in the clonal cell lines and pituitary corticotrophs should help identify the particular clonal cell line most appropriate for addressing a specific question. Investigations utilising these cell lines should allow more accurate deduction of signalling mechanisms.

5.4 FUTURE WORK

As discussed above, the clonal cell lines will need to be characterised to a greater extent before they are used to determine the molecular mechanism of desensitisation of the V1bR. In order to more fully characterise these clonal cell lines a number of experiments are required including:

1) receptor binding assays using [^{3}H]AVP to determine the number of receptors present at the plasma membrane of each cell line,
2) measurement of intracellular Ca^{2+} changes in response to stimulation with AVP, and
3) growth curves for all four of the clonal cell lines

Receptor binding assays will provide information on the total numbers of receptors present at the plasma membrane of the clonal cell lines. This will allow determination of whether the clonal cell lines are expressing the receptor at different levels. [^{3}H]AVP has been used previously for receptor binding studies with the rV1bR in both anterior pituitary tissues (Aguilera et al., 1994) as well as immortalised cell cultures (Orcel et al., 2009; Young et al., 2007). Specifically Orcel et al., (2009) used receptor binding assays to determine the level of receptors present at the plasma membrane in transfected HEK293 cells. Therefore, this approach is likely to be most compatible with the developed model cell system as it also used HEK293 cells.

Examining the levels of intracellular Ca^{2+} will allow further analysis of whether signalling is occurring normally within the clonal cell lines. Fluorescence microscopy has been utilised recently to examine changes in intracellular Ca^{2+} levels with the GPCR protease activated receptor 2 (Gu and Lee, 2010) and IP_3 stimulated intracellular Ca^{2+} levels in response to mechanical stimulation (Nezu et al., 2010) as well as with the rV1bR (Young et al., 2007). Young et al., (2007), examined the changes in intracellular Ca^{2+} levels of the wild type rV1bR as well as various tagged forms of the receptor in response to 10 nM AVP in CHO cells. This research used the rat V1bR, transiently transfected into CHO cells. The results from that

research should be able to provide a comparison for changes in intracellular Ca^{2+} levels in the clonal cell lines developed in this research.

As differences in the growth rates of the clonal cell lines were observed during passaging, it would be useful to generate growth curves for each of the lines. This would provide specific details on the growth phases for each clonal cell line. It would also provide an indication of the disruption caused to the individual lines through plasmid DNA integration into the genome. The growth curves could be compared with those developed for the parental (untransfected) HEK293 cell line. This would provide information on how disruptive the transfection event had been to the cell growth rate and therefore the cell cycle and general overall health of the cell. This experiment would not provide any further information on cell signalling.

Along with the AVP responsiveness and desensitisation data, these future experiments will provide a more complete picture of signalling in the clonal cell lines. This will provide a base line for work analysing the molecular mechanisms of desensitisation.

The characterised clonal cell lines will then be used in our laboratory to determine the molecular mechanism of rV1bR desensitisation. Initially this will involve using siRNA to knock down GRKs -2, -3, -5, and -6. The success of the various knock downs can be determined using Western blots and quantitative reverse transcriptase PCR measurements of GRK protein and mRNA respectively. The protocols for these have already been developed in this laboratory for GRK5 by Gatehouse (2008) and as yet unpublished MSc work by Katherine van Bysterveldt. The effect of these knock downs on desensitisation will be examined using the IP assay and therefore, the effect that knock down of individual GRKs has on IP production in response to AVP stimulation. This work will most likely require alterations to the IP assay protocol used in this research. It is most likely that AVP responsiveness will be measured over a time course, in both control cells and in those where expression of a specific GRK has been knocked down. If the knocked down GRK was involved in the desensitisation process, IP accumulation would be expected to increase to a higher level over the time course as all of the receptors would continue to transduce the external signal. In contrast in the control cells the rate of IP accumulation would drop over N.CITE
provide information concerning the molecular mechanisms involved in V1bR desensitisation.

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APPENDIX A:

MATERIALS

A.1 REAGENTS

AG1-X8 anion-exchange resin	BioRad Laboratories
Agarose	Gibco, Invitrogen Corporation
Amine coated 24 well plates	Becton Dickinson Biosciences
Ammonium formate	Sigma-Aldrich Company
Ampicillin	Sigma-Aldrich Company
Arginine Vasopressin Powder	Sigma-Aldrich Company
AxyPrep™ Plasmid Miniprep Kit	Axygen Biosciences
Bacteriological Agar	Germantown
Biodegradable Counting Scintillant	Amersham Biosciences
Bovine Serum Albumin	Gibco, Invitrogen Corporation
Bromophenol Blue	BDH Laboratory Supplies
Buffer O (10x)	Fermentas Life Sciences
Carboxyl coated 24 well plates	Becton Dickinson Biosciences
Concentrated Hydrochloric Acid	BDH Laboratory Supplies
Competent DH5 alpha *E. coli* cells	Invitrogen Corporation
Dimethyl Sulfoxide	Sigma-Aldrich Company
Dulbecco's modified Eagles medium (-inositol)	MP Biomedical
Ethanol (100%)	BDH Laboratory Supplies
Foetal Bovine Serum	Gibco, Invitrogen Corporation
Formic Acid	Sigma-Aldrich Company
FuGENE 6	Roche Applied Science
G418 Powder	Gibco, Invitrogen Corporation
Glycerol	BDH Laboratory Supplies
GlutaMAX™	Gibco, Invitrogen Corporation
High DNA Mass Ladder	Gibco, Invitrogen Corporation
Human Embryonic Kidney 293 cell line	ATCC
Hydrochloric acid	BDH Laboratory Supplies

Isopropanol	BDH Laboratory Supplies
Kanamycin Sulfate	Acros Organics
L-ascorbic acid	Sigma-Aldrich Company
L-glutamine	Gibco, Invitrogen Corporation
LB Broth Miller (Luria Bertani)	Becton Dickson Biosciences
LB Broth Base (Lennox Broth Base)	Invitrogen Corporation
Lambda DNA/PstI Marker, 24	Fermentas Life Sciences
Lithium Chloride	Sigma-Aldrich Company
Magnesium Chloride	BDH Laboratory Supplies
Minimal Essential Medium (MEM)	Gibco, Invitrogen Corporation
myo-[^{3}H]inositol	Amersham Biosciences
MYO-INOSITOl,[^{3}H]	Perkin Elmer Inc.
NotI Restriction Enzyme	Fermentas Life Sciences
OPTIPHASE 'HISAFE' 3 scintillantion cocktail	Perkin Elmer Inc.
Penicillin/Streptomycin	Gibco, Invitrogen Corporation
Poly-D-Lysine coated 24 well plates	Becton Dickinson Biosciences
Polyprep Chromatography Columns	BioRad Laboratories
Primaria coated 24 well plates	Becton Dickinson Biosciences
pUC19 plasmid DNA	Invitrogen Corporation
PureLink™ HiPure Plasmid Filter Purification Kits	Invitrogen Corporation
Recovery™ Cell Culture Freezing Medium	Invitrogen Corporation
SalI Restriction Enzyme	Fermentas Life Sciences
Sodium bicarbonate	BDH Laboratory Supplies
Sodium chloride	BDH Laboratory Supplies
Sodium formate	Sigma-Aldrich Company
Sodium pyruvate	Gibco, Invitrogen Corporation
Sodium tetra borate	Sigma-Aldrich Company
Standard cell culture 24 well plates	Thermo Fischer Scientific
Subcloning Effciency™ DH5α™ Competent Cells	Invitrogen Corporation
SYBR® Safe DNA Gel Stain 10,000X	Invitrogen Corporation
T4 DNA Ligase	Fermentas Life Sciences
T4 DNA Ligase Buffer	Fermentas Life Science
Tris-HCl TRIZMA Base	Sigma-Aldrich Company

Trypan Blue	BDH Laboratory Supplies
Trypsin/EDTA (T/E)	Gibco, Invitrogen Corporation
XhoI restriction enzyme	Gibco, Invitrogen Corporation
Zymoclean™ Gel DNA Recovery Kit	ZymoResearch Corporation

APPENDIX B:

SOLUTIONS AND MEDIA

B.1 CELL CULTURE

B.1.1 MINIMAL ESSENTIAL MEDIUM (MEM)

One packet of commercially prepared powder for 1 L MEM was dissolved in Nanopure H_2O. Sodium bicarbonate ($NaHCO_3$) (1.5 g) and 10 ml of sodium pyruvate (final concentration 110 mg/l) were added. The solution was then made up to 1 L with Nanopure H_2O and the pH adjusted to 7.1. The solution was filter sterilised with a 0.45 μm membrane filter, and the medium was checked for sterility. The medium was stored at 4°C for no more than three months. For culturing HEK293 cells the medium was supplemented with 10% FBS (v/v). For selecting stable clones it was also supplemented with 500 μg/ml G418. For the maintenance of stable clones it was supplemented with 250 μg/ml G418. After three weeks of storage the medium was supplemented with GlutaMAX™ (final concentration 2 mM). When required the medium was also supplemented with penicillin (final concentration 100 U/ml)/streptomycin (final concentration 100 μg/ml).

B.1.2 PHOSPHATE BUFFERED SALINE (PBS)

To make a 5x stock solution the reagents below were dissolved in Nanopure H_2O and the solution was made up to 200 ml. The pH was adjusted to ~7.1. To make a 1x working solution the same weight of chemicals was made up to 1 L with Nanopure H_2O. The pH was then adjusted to 7.4. The solutions were stored room temperature, and discarded if any precipitate or growth appeared.

KH_2PO_4	0.2 g
KCL	0.2 g
NaCl	8.0 g
Na_2HPO_4	1.15 g (or 2.2 g $Na_2HPO_4.7H_2O$)

B.1.3 TRYPAN BLUE STOCK SOLUTION

A 2% trypan blue stock solution was made by dissolving 0.02 g of trypan blue (BDH) in 10 ml of Nanopure H_2O. This solution was filtered into a clear glass vial through a 0.22 μm filter. The filtered solution was stored at 4°C and discarded if a precipitation or growth appeared.

B.1.4 TRYPAN BLUE WORKING SOLUTION

Trypan blue working solution was made by adding 4 parts of trypan blue stock solution to 1 part 5x PBS in a 4 ml polystyrene tube. The working solution was made up fresh as required for cell counts.

B.1.5 FREEZING MEDIUM

To freeze the HEK293 cells for cryogenic storage two different freezing mediums were used over the course of this research. The first was a commercially prepared medium from Invitrogen, Recovery™ Cell Culture Freezing Medium. The second was prepared entirely the laboratory.

To prepare cells at ~80% confluence from a 75 cm^2 flask for freezing as four 1 ml aliquots, the following solutions were initially mixed.

MEM-	1.2 ml
DMSO	0.4 ml
FBS	0.4 ml

These above solutions were mixed in a glass container because concentrated DMSO can permeate plastic. The cell pellet was re-suspended in 2 ml of MEM-. The above 2 ml solution was then slowly added to the cell suspension and gently mixed. The final concentration of DMSO was 10%, and the final concentration of FBS was 10%. The cells were then aliquoted into 1 ml cryotubes for freezing. The volumes the freezing medium were halved in order to freeze two tubes of cells at ~80% confluence as 1 ml aliquots from a 25 cm^2 flask.

B.2 PREPARATION OF PLASMID DNA FOR TRANSFECTION

B.2.1 TBE AGAROSE (0.7%) GEL

The agarose was dissolved into 200 ml of 0.5x TBE buffer. This mixture was heated in order to dissolve the agarose. Once the agarose was dissolved SYBR® Safe was added. The gel was allowed to cool slightly before adding it to the box. Enough of the gel mixture was poured into the box to ensure that approximately 5 mm of the comb was covered. The gel was allowed to set and was run using 0.5x TBE as a running buffer.

Agarose	1.4 g
SYBR® Safe	20 µl

B.2.2 TBE BUFFER 0.5x

The reagents listed below were dissolved in 1 L of Nanopure H_2O to make a 5x stock solution. The pH of the resulting solution was adjusted to 8.0 and then stored at 4°C. The 5x stock solution was diluted 1:9 in Nanopure ddH_2O to produce the 0.5x working solution.

Tris-Base	54 g
Boric Acid	27.5 g
0.5 M EDTA	20 ml

B.2.3 LB BROTH

B.2.3.1 Culture Broth

LB broth powder with different compositions was sourced from two different manufacturers, Becton Dickson Biosciences and Invitrogen Corporation. The instructions on how both of these were made are described below.

B.2.3.1.1 LB Broth Miller

To prepare LB Broth Miller (Luria Bertani) sourced from Becton Dickson Biosciences, 25 g of powder were dissolved in 1 L of Nanopure H_2O. The resulting broth solution was sterilised by autoclaving. The broth solution has the following final composition:

Tryptone	10 g/L
Yeast Extract	5 g/L
NaCl	10 g/L

When required, antibiotics were added to the broth after sterilisation.

B.2.3.1.2 LB Broth Base (Lennox Broth Base)

To prepare LB broth (Lennox Broth Base) sourced from Invitrogen, 20 g of powder were dissolved in 1 L of Nanopure H_2O. The resulting broth solution was sterilised by autoclaving. The broth solution has the following final composition:

SELECT Peptone 140	10 g/L
SELECT Yeast Extract	5 g/L
NaCl	10 g/L

When required, antibiotics were added to the broth after sterilisation.

B.2.3.2 Agar Plates

Bacteriological agar (Germantown) (15 g) was added to 1 L of broth prior to autoclaving. Plates were poured after the solution was sterilised. To do this the agar/broth was heated in the microwave to dissolve the agar. The solution was cooled to ~50°C before pouring the plates (~25 ml per plate). If antibiotics were required they were added just prior to pouring. The plates were left to set for 25 min with the lids on. Once set, the plates were turned upside down with the bases propped open on the lids, which were facing upwards, to allow the vapour to escape for ~10 min. The lids were then turned face down and the plates, with the upside down bases propped open on the lids, were then left to dry for ~4-5 hours. Once dry, the plates were wrapped in plastic wrap, then with aluminium foil and stored upside down at 4°C. The plates were kept for no more than 1-2 weeks before use.

B.2.4 KANAMYCIN STOCK SOLUTION

Kanamycin was received in a commercially prepared powdered form. The required amount was weighed and made up to 40 mg/ml with the appropriate volume of Nanopure H_2O. The solution was filter-sterilised through a 0.22 μm filter. The sterile solution was aliquoted and stored at -20°C. Working solutions of kanamycin were created by diluting appropriately in culture broth.

B.2.5 TE8

The reagents listed below were dissolved in 100 ml of Nanopure H_2O. This gave the final concentrations of Tris-HCl 10 mM and EDTA 1 mM. The solution's pH was adjusted to 8.0 and then sterilised by autoclaving. The sterile solution was stored at room temperature.

Tris-HCl (TRIZMA Base) (pH 8.0)	0.121 g
EDTA	0.037 g

B.2.6 REact 2 BUFFER 10x

The reagents listed below were dissolved in 25 ml of Nanopure H_2O. The initial pH had to be lowered slightly before the chemicals would dissolve. Once the reagents were dissolved the solution's pH was adjusted to 8.0. It was aliquoted into 1.7 ml microfuge tubes in 1 ml lots and store at -20°C until needed.

Tris-HCl (TRIZMA Base), pH 8.0	1.51 g
MgCl	0.51 g
NaCl	0.73 g

B.2.7 AMPICILLIN STOCK SOLUTION

Ampicillin was received in a commercially prepared powered form. The required amount was weighed and made up to 50 mg/ml with the appropriate volume of Nanopure H_2O. The solution was filter-sterilised through a 0.22 μm filter. The sterile solution was aliquoted and stored at 4°C. The working solutions of ampillicin were created by diluting appropriately in culture broth.

B.2.8 0.8% AGAROSE GEL

Agarose was added to 50 ml of 0.5x TBE buffer. SYBR® Safe was added and the solution was heated in the microwave until all the agarose had dissolved. The solution was allowed to cool to about ~50°C before pouring. The gel took ~30 min to set. The running buffer for the gel was 0.5x TBE.

Agarose	0.4 g
TBE	0.5x
SYBR® Safe DNA Gel Stain 10,000x	1:10,000 dilution

B.2.9 GEL LOADING DYE 6x

The reagents listed below were added to Nanopure H_2O to give the final concentrations listed. A total volume of 12 ml was made and the solution was aliquoted and stored at 4°C.

Bromophenol Blue	0.25%
Xylene Cyanol	0.25%
Glycerol	30%

B.3 OPTIMISATION OF ANTIBIOTIC SELECTION CONDITIONS

B.3.1 G418 STOCK SOLUTION

G418 was received in commercially prepared powder form. The required amount was weighed and made up to 50 mg/ml with the appropriate volume of Nanopure H_2O. The solution was filter-sterilised through a 0.22 μm filter. The sterile solution was aliquoted and stored at 4°C. The solution can be stored at this temperature for up to two years. Working solutions of G418 were created by diluting appropriately in MEM+.

B.4 INOSITOL PHOSPHATE (IP) ASSAY

B.4.1 LABELLING SOLUTION

The BSA and GlutaMAX™ were mixed with an appropriate volume of DMEM without inositol to give the concentrations below. The solution was then filter sterilised through a 0.22 μm filter, and placed in the CO_2 incubator at 37°C to equilibrate. Just prior to labelling the wells the *myo*-[^{3}H]inositol was added to the solution to give the below concentration.

BSA	0.1%
GlutaMAX™	1%
myo-[^{3}H]inositol	2.5 μl/ml

B.4.2 LITHIUM CHLORIDE (LiCl) 1 M

LiCl was dissolved into Nanopure H_2O to make a 1 M stock solution. This stock solution was filtered sterilised into a sterile glass vial through a 0.22 μm filter, and stored at 4°C.

B.4.3 AVP STOCK SOLUTION

AVP was received in a commercially prepared powder form ([Arg^8]-vasopressin, acetate salt). The required amount was weighed and made up to 20 μM with the appropriate volume of MEM containing 0.3% BSA and 0.005% L-ascorbic acid. The stock solution was aliquoted and stored at -80°C. The stock solution was diluted in MEM- to give the desired concentrations for IP assays.

B.4.4 DETERMINATION OF AVP RESPONSIVENESS OF TRANSFECTED CELLS

B.4.4.1 Basal solution

The reagents listed below were mixed in MEM- to obtain the concentrations below. The solution was equilibrated to 37°C, by placing it in a CO_2 incubator.

BSA	0.1%
LiCl 1 M stock solution	10 mM

B.4.4.2 Stimulation solution

AVP stock solution (20 μM) was diluted to 100 nM with basal solution. The AVP was added to the solution just prior to use, or if added earlier in the day it was stored in the refrigerator until 30 min prior to use, when it was equilibrated to 37°C in a CO_2 incubator.

B.4.5 DETERMINATION OF AVP RESPONSIVENESS AND MAGNITUDE OF DESENSITISATION OF TRANSFECTED CELLS

B.4.5.1 Control solution

The BSA was mixed into an appropriate amount of MEM- to give the below concentration of BSA. The solution was equilibrated to 37°C, by placing it in a CO_2 incubator.

BSA	0.1%

B.4.5.2 AVP pre-treatment solution

AVP stock solution (20 μM) was diluted to 100 nM with control solution. This 100 nM AVP solution was diluted to 5 nM with control solution to give the pre-treatment solution. The AVP was added to the solution just prior to use, or if added earlier in the day it was stored in the refrigerator until ~30 min prior to use, when it was equilibrated to 37°C in a CO_2 incubator.

B.4.6 "STOP" SOLUTION

The chemicals were dissolved in an appropriate volume of Nanopure H_2O, to give the concentrations below. The solution was stored at 4°C.

KOH	1 M
Sodium Borate	18 mM
EDTA	3.8 mM
Sodium hydroxide	7.6 mM

B.4.7 "NEUTRALISATION" SOLUTION

Hydrochloric acid (37%) was mixed with Nanopure H_2O to give a 7.5% HCl solution. Five ml of this solution was combined with 5 ml of the "stop" solution and 5 ml of MEM. The pH of the mixture was measured and adjusted to 7.0 by addition of a known volume of Nanopure H_2O. The "neutralisation" solution was then adjusted by adding the appropriate volume of Nanopure H_2O so that when the three solutions were combined in equal volumes the resulting solution would have a neutral pH. The neutralisation solution was stored at 4°C.

B.4.8 ELUTION BUFFER II

The solution was made up fresh on the final day of the IP Assay. The reagents were dissolved in an appropriate volume of Nanopure H_2O to give the concentrations below.

Sodium borate	5 mM
Sodium formate	60 mM

B.4.9 ELUTION BUFFER VI

The solution was made up fresh on the final day of the IP Assay. The reagents were dissolved in an appropriate volume of Nanopure H_2O to give the concentrations below.

Formic Acid	100 mM

Ammonium Formate	2 M

B.4.10 REGENERATION SOLUTION

The solution was made up fresh on the final day of the IP Assay. The reagents were dissolved in an appropriate volume of Nanopure H_2O to give the concentrations below.

Formic Acid	100 mM
Ammonium Formate	3 M